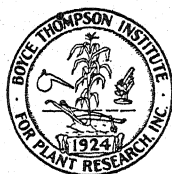


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# ERRATA

Page	7, line 16, for '38 mm.'	read '38 cm.'			
"	16	"	15	"	' <i>Lycopersicum</i> ' read ' <i>Lycopersicon</i> '
"	163	"	9	"	'commerical' " 'commercial'
"	197	"	2	"	' <i>Lycopersicum</i> ' " ' <i>Lycopersicon</i> '
"	200	"	15	"	" " "
"	222	"	43	"	" " "
"	310	"	12	"	'per cents' " 'per cent'



SOME FACTORS AFFECTING THE EFFICIENCY OF CONTACT  
INSECTICIDES. I. SURFACE FORCES AS RELATED TO  
• WETTING AND TRACHEAL PENETRATION<sup>1</sup>

FRANK WILCOXON AND ALBERT HARTZELL

INTRODUCTION

The importance of surface forces in the performance of contact insecticides has long been recognized. Cooper and Nuttall (3) discussed this question and gave a method of estimating the wetting power of a spray solution by determination of its interfacial tension against an oil adopted as standard. Woodman (16) used the area covered by a known amount of spray applied to a glass plate as a criterion of its ability to wet leaves and spread over them. Hamilton (5) made use of a similar method but considered the time required to spread over a standard surface as an indication of the efficiency of the spray. Moore and Graham (14) investigated the relation between the physical properties and the efficacy of contact insecticides and made a distinction between the wetting of a solid surface by a liquid and the spreading over the surface. This distinction appears to be determined by the value of the contact angle between a drop of spray liquid and the insect integument.<sup>2</sup> If this angle is  $180^\circ$ , neither wetting nor spreading takes place. If the angle has some value between  $0^\circ$  and  $180^\circ$ , then wetting takes place, but the liquid does not spread down to a film, while if the contact angle has a value of  $0^\circ$ , both wetting and spreading have taken place. Most aqueous spray solutions exhibit a contact angle between  $0^\circ$  and  $180^\circ$ , and do not spread to a film when drops are carefully placed on the integument of insects. Instead of attempting to predict the behavior of spray fluids from measurements of surface tension and contact angles, Moore made observations on the penetration of a large number of liquids into the tracheal system of the cockroach and several other species. He found that no aqueous solution penetrated the tracheae except soap solutions. A historical review of work on spreading and wetting of insecticides prior to 1921 is presented by Moore (13).

The phenomena that take place when a solid is in contact with a liquid and with air are of great interest in many lines of investigation which at first sight seem to have little in common. For example, in the flotation process for concentration of ores, advantage is taken of the selective wetting of the mineral particles in separating the ore from the gangue (15).

<sup>1</sup> Herman Frasch Foundation for Research in Agricultural Chemistry. Paper No. 14.

<sup>2</sup> In this discussion the angle of contact is the angle between the tangent to the surface of the liquid at the point where air, liquid, and solid meet, and the tangent to the solid surface at that same point. The angle lies within the liquid.

Again in the paint industry the surface forces operating between the pigment and the vehicle are of importance in determining the properties of the paint (7).

Harkins and Feldman (8) in their study of the spreading of liquid over liquid have derived by thermodynamic reasoning a quantity called the "spreading coefficient" which they showed to be a valid criterion of spreading or non-spreading of one liquid over another. The spreading coefficient is simply the difference between the work of cohesion  $W_c$ , of liquid  $A$  and the work of adhesion  $W_a$ , between liquid  $A$  and liquid  $B$ . If the work of adhesion is greater than the work of cohesion, spreading takes place, otherwise it does not take place. That these considerations may be applied at least in part to solid surfaces is indicated by Adam and Jessop (1), who determined the work of adhesion between water and a number of different solid surfaces. The work of adhesion  $W_a$  is equal to the surface tension of the solid against air, plus the surface tension of the liquid against air, minus the surface tension of the solid against the liquid. It is the work done in pulling a column of liquid 1 cm.<sup>2</sup> in cross-section, away from the solid surface.

$$W_a = T_{SV} + T_{LV} - T_{SL} \quad (\text{Equation 1})$$

The only one of these quantities which is susceptible to measurement is the surface tension of the liquid against air. However, in the cases where the liquid does not spread over the surface but forms a definite angle of contact, as is the case with most aqueous spray solutions, the other two tensions may be eliminated by combining Equation 1 with the equation of equilibrium of a liquid resting on a solid surface having a contact angle  $\theta$ . This gives the work of adhesion in terms of the surface tension of the liquid against air and the cosine of the contact angle.

Thus:

$$W_a = T_{SV} + T_{LV} - T_{SL} \quad (\text{Equation 1})$$

$$T_{LV} \cos \theta = T_{SV} - T_{SL} \quad (\text{Equation 2})$$

giving on subtraction

$$W_a = T_{LV} (1 + \cos \theta) \quad (\text{Equation 3})$$

The work of cohesion  $W_c$  is equal to twice the surface tension of the liquid against air, that is,  $2T_{LV}$ . It is the work done in pulling apart a column of liquid 1 cm.<sup>2</sup> cross-section (7). The spreading coefficient defined above becomes then:

$$\begin{aligned} \text{Spreading coefficient} &= W_a - W_c \\ &= T_{LV} (\cos \theta - 1) \quad (\text{Equation 4}) \end{aligned}$$

All that is necessary to evaluate the spreading coefficient then is to determine the surface tension of the liquid against air, and to measure the con-

tact angle of the liquid on the solid, in this case the integument of the insect.

In the present paper it was attempted to rate various spray solutions with respect to their tendency to spread on insect integument and by means of toxicity experiments with *Aphis rumicis* L. find out to what extent spreading is correlated with toxicity.<sup>3</sup>

#### PRELIMINARY OBSERVATIONS

Nasturtium leaves infested with *Aphis rumicis* were placed on the stage of a binocular microscope and sprayed with a number of different solutions while under observation. In the case of most of the solutions the drops of spray wetted the insect rather poorly. With water the drops retained their spherical form (Fig. 1, A), but did not spread and rapidly evaporated. Solutions containing gelatin, saponin, or calcium caseinate gave somewhat flatter drops, but did not wet the insect thoroughly. Only in the case of soap solutions containing 0.5 per cent or more of soap were many of the drops observed to flatten out and spread to a thin film. It appears then that most spray solutions in contact with insect integument exhibit a contact angle greater than zero, which involves a negative value of the spreading coefficient.

Excised portions of the tracheae from the common tomato worm (*Phlegethontius quinquemaculata* Haworth) were submerged at one end in drops of spray on a microscope slide and observed under a low power. It was found that water and several other spray solutions did not enter the tracheae, the surface forces between the liquid and the tracheal wall being evidently opposed to the hydrostatic pressure tending to cause entrance. This is quite different from the behavior of such solutions in capillary glass tubes. Soap solutions containing 0.5 per cent or more of soap entered the tracheae (Fig. 1, B), and exhibited a contact angle within the tracheal walls of slightly less than 90°. This large contact angle indicates that even in the case of soap solutions the capillary forces tending to cause entrance were relatively feeble. The height  $h$  to which a liquid of surface tension  $T$  will rise in a capillary of radius  $r$ , when the liquid exhibits a contact angle  $\theta$ , with the walls of the capillary, is given by Edser (4)

$$h = \frac{2T \cos \theta}{rg}$$

It has generally been assumed that the intima of tracheae is chitinous, but Campbell (2) was unable to demonstrate the presence of chitin in the tra-

<sup>3</sup> While this work was in progress a bulletin by W. C. O'Kane et al. on "Surface tension, surface activity, and wetting ability as factors in the performance of contact insecticides. Studies of contact insecticides I" (New Hampshire Agric. Exp. Sta. Tech. Bull. 39) was published which treats of certain phases of wetting and penetration of tracheae by sprays.



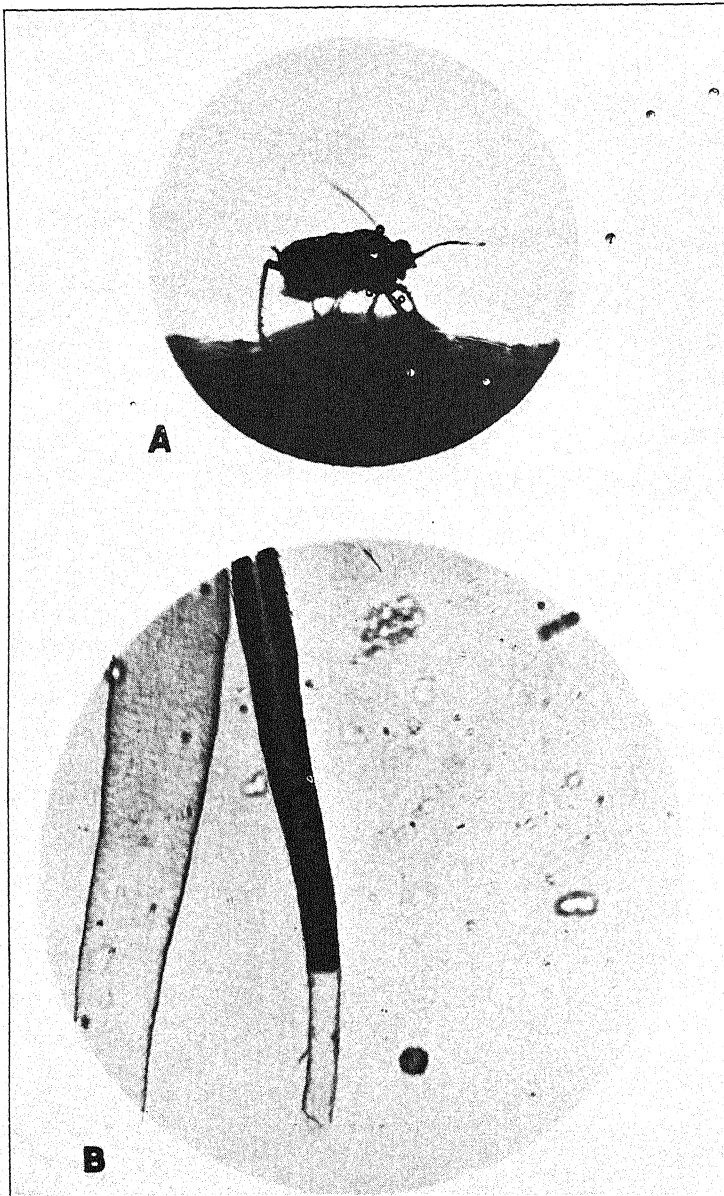


FIGURE 1. A. An aphid sprayed with water. Note that the drops retain their spherical form. They evaporated rapidly and did not spread. B. Excised tracheae submerged in soap solution. The contact angle within the walls, it will be noted, is slightly less than  $90^\circ$ . Note the flatness of the meniscus, indicating that capillary forces are negligible.

cheae of the honey bee and the house fly although it could be detected in the American cockroach.

#### SPREADING COEFFICIENT AND TOXICITY OF NICOTINE SOLUTIONS

In order to determine the extent to which spreading and toxicity are correlated, the following series of experiments was carried out. A solution containing 0.1 per cent of free nicotine was prepared. To portions of this were added calcium caseinate, Penetrol (a proprietary product) (9), and sodium oleate in such amounts that the final solutions each contained 0.5 per cent of the spreading agent. These solutions were tested for toxicity against *Aphis rumicis*. The spreading coefficients of these solutions were determined by surface tension measurements and by the determination of the contact angle of each solution on the integument of freshly-killed adult Colorado potato beetles (*Leptinotarsa decemlineata* Say) and Mexican bean beetles (*Epilachna corrupta* Muls). The measurement of the surface tension was carried out by the drop weight method as described by Harkins and Brown (6), and the corrections given in International Critical Tables (4: 435) were applied. The glass tip used for forming the drops was made by cutting off the tip from a Traube stalagmometer. The time of forming the drop was three minutes. In dealing with colloidal solutions particularly, the surface tension varies with the time elapsed since the formation of the surface, and it was considered best to adopt a uniform time of drop formation. The difficulty involved in accurately determining the surface tension of soap solutions has been discussed by Johlin (10). When a new surface is formed, as for example, when a column of liquid is broken into drops by passage through a nozzle, considerable time elapses before adsorption equilibrium is reached between the surface and interior of the drop. This time is longer in the case of colloidal solutions than in the case of true solutions. The angle of contact was estimated by photographing drops of spray liquid placed on the integument of the insect (Fig. 2, A, B, C, D). The plates were placed in an enlarging camera and tracings made of the outline of the drop and that of the insect. From these tracings the angle of contact was estimated by drawing tangents. This procedure gave the so-called advancing angle of contact. It was not found possible to obtain a high degree of reproducibility in the determination of the contact angle. For example, the average deviation from the mean in one series of ten measurements on the same solution was 8°. For the receding angle the following procedure was adopted. The insect was immersed in the spray liquid and a pipette full of air introduced above and a few mm. from the insect. An air bubble was partially expelled from the pipette in such a way that it made contact with the integument of the insect (Fig. 2, E, F). Under these conditions the air bubble displaces the liquid to a greater or lesser degree, and a contact angle is formed between liquid, air bubble, and insect

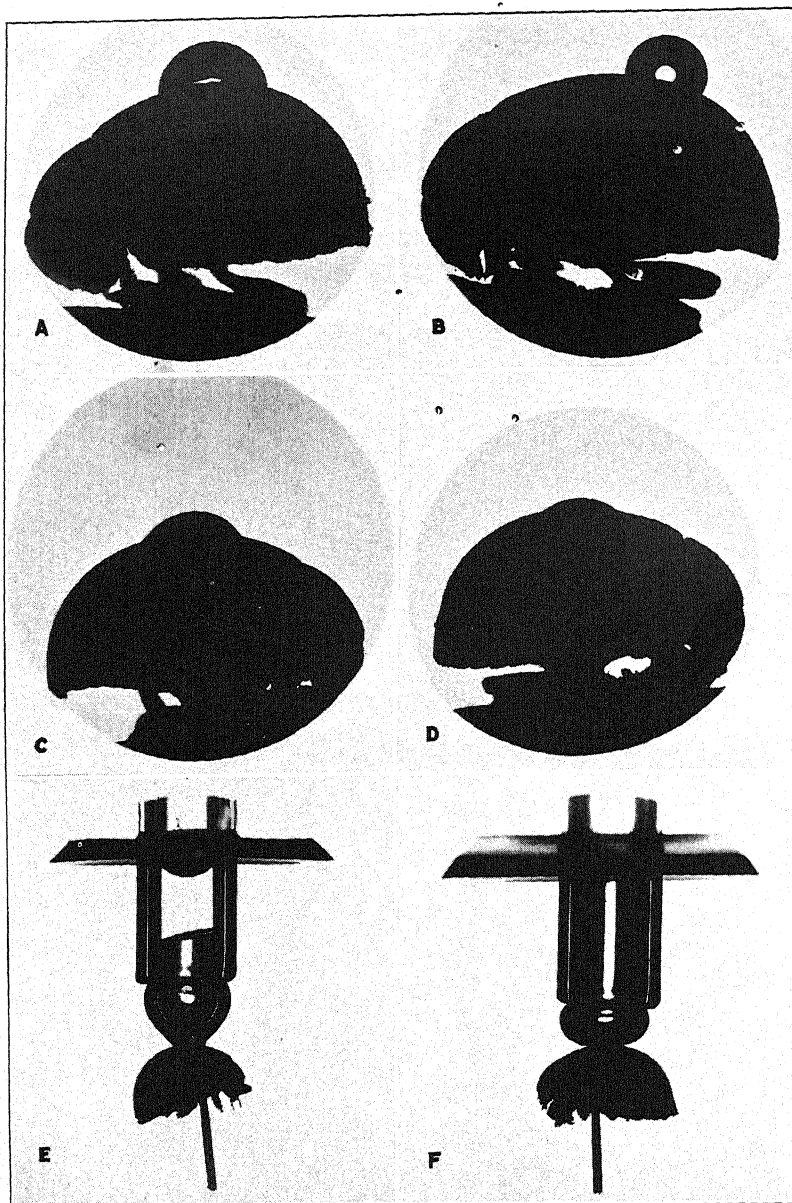


FIGURE 2. Drops of liquid on the integument of Colorado potato beetle: A. water, B. 0.5% calcium caseinate solution, C. 0.5% Penetrol solution, D. 0.5% soap solution. Air bubbles in contact with integument of Mexican bean beetle submerged: in E. 0.1% nicotine solution, showing that the air pushes back the liquid when the liquid does not wet the insect well, and F. in sodium oleate solution which wets well. In the latter case the bubble does not push back the liquid.

integument, whose value depends on the surface forces involved, and is generally less than the advancing angle previously measured. The true equilibrium angle lies between the advancing and receding angles. According to Adam and Jessop (1), the cause of this hysteresis of the contact angle lies in the friction between the liquid and solid. They calculate the equilibrium angle by means of the following relation:

$$\cos \theta = \frac{\cos \theta_a + \cos \theta_r}{2}$$

where  $\theta_a$  and  $\theta_r$  refer to the advancing and receding angles. From the value of the contact angle obtained in this way, and the surface tension of the liquid used, the spreading coefficient was calculated by Equation 4. These coefficients have a negative value, and the nearer the coefficient approaches zero, the better the solution as regards spreading.

TABLE I  
THE SURFACE TENSION, CONTACT ANGLE, AND SPREADING COEFFICIENT OF  
THE SOLUTIONS TESTED

Solution	Surface tension at 29° C.	Equilibrium contact angle	Spreading coefficient
0.1% nicotine in distilled water	68 dynes	101°	-81
0.5% calcium caseinate with 0.1% nicotine.....	52 dynes	49°	-18
0.5% Penetrol with 0.1% nico- tine.....	39 dynes	28°	- 4.8
0.5% sodium oleate with 0.1% nicotine.....	23 dynes	29°	- 2.9

The toxicity experiments were carried out in the following manner. Nasturtium plants infested with agamic individuals of *Aphis rumicis* were sprayed with each of the solutions whose spreading coefficient was to be determined with a DeVilbiss atomizer No. 15 nozzle attached to a compressed air line whose pressure was maintained at 38 mm. of mercury. Each plant received 35 cc. of spray solution. The plants were rotated during the spraying by hand and care was taken to get uniform coverage. The plants were placed in an insectary on strips of paper with tanglefoot barriers at a temperature of 72°-84°F. and a relative humidity of 60-82 per cent. Counts were made after an interval of 24 hours. All individuals that reached the tanglefoot barrier were counted as alive. The individuals on the plant and on the paper were examined under a binocular and the doubtful cases were probed with a needle. Those individuals which moved a leg or antenna were counted as alive. The experiment was run in duplicate and at least 500 insects were counted for each test. The results of these tests appear in Table II. The order of effectiveness of the four solutions tested is the same as that indicated by their spreading coefficients.

TABLE II  
TOXICITY OF THE SOLUTIONS TESTED TO APHIS RUMICIS

Solution	% dead Exp. A	No. counted	% dead Exp. B	No. counted
0.1% nicotine in distilled water...	60.3	559	61.6	579
0.5% calcium caseinate with 0.1% nicotine.....	81.3	337	90.2	683
0.5% Penetrol with 0.1% nicotine..	92.2	734	93.3	791
0.5% sodium oleate with 0.1% nico- tine.....	96.2	536	97.4	745

#### TRACHEAL PENETRATION

In order to follow more closely the effect of spreading agents on the efficiency of contact insecticides a study was made of the actual penetration of the four solutions used above into the tracheae of full grown larvae of the common tomato worm (*Phlegethontius quinquemaculata*). The larva was submerged in a beaker containing the spray solution to a depth of three inches for a period of one minute. The specimen was then removed and the exterior rinsed from a wash bottle and then pinned to a small dissecting dish with a wax bottom colored with lampblack. With the aid of a binocular microscope a longitudinal incision was made with curved scissors ventrad of the spiracles, care being taken not to sever the main tracheal branches; the integument was then pinned back exposing the tracheae radiating from the spiracles. Figure 3, A, illustrates the type of dissection made. Dissections were made without the use of water or other liquids. It could readily be observed whether or not penetration had taken place (Fig. 3, B). Immediately after dissection photomicrographs were taken with incident light using the simple microscope in a large Zeiss photomicrographic outfit.

In the following experiments, owing to the dissections involved, we were necessarily dealing with small samples, and therefore each experiment was repeated from three to five times to insure a reasonable certainty that the observation was significant and the investigation was extended over two seasons. The observations were further substantiated by a series of photomicrographs, a number of which are here presented. Between 100 and 200 dissections were made in the course of this investigation.

In the case of 0.1 per cent nicotine solution without spreader as well as the solution containing 0.5 per cent calcium caseinate no penetration was observed. However, solutions containing 0.5 per cent Penetrol and also one containing 0.5 per cent sodium oleate showed penetration from one-third to two-thirds the length of the larger tracheae. Observation of a number of individuals showed somewhat better penetration for sodium oleate than for Penetrol. As the Penetrol and sodium oleate were progressively diluted, penetration was evident with sodium oleate at greater dilutions than with Penetrol. Careful observation of the meniscus formed by sodium oleate in

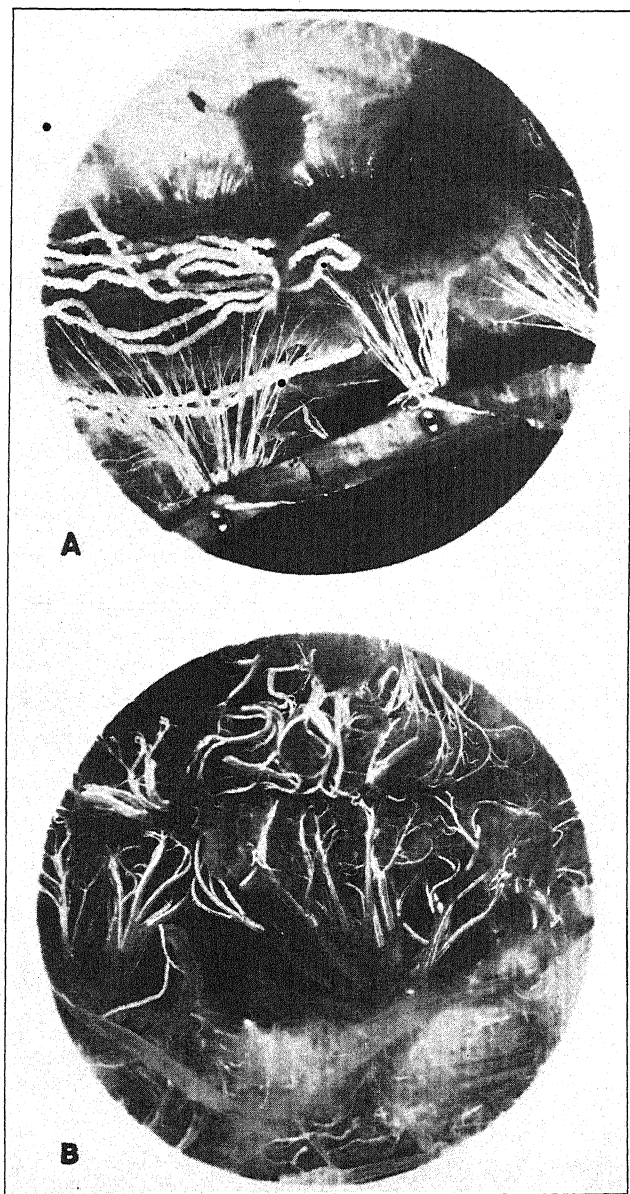


FIGURE 3. A. Abdominal region of larva of tomato worm showing type of dissection used in experimental work. Note the tracheae radiating fan-like from the spiracles. B. The tracheal tubes, it will be noted, are partly filled with a spray solution containing soap and nicotine.

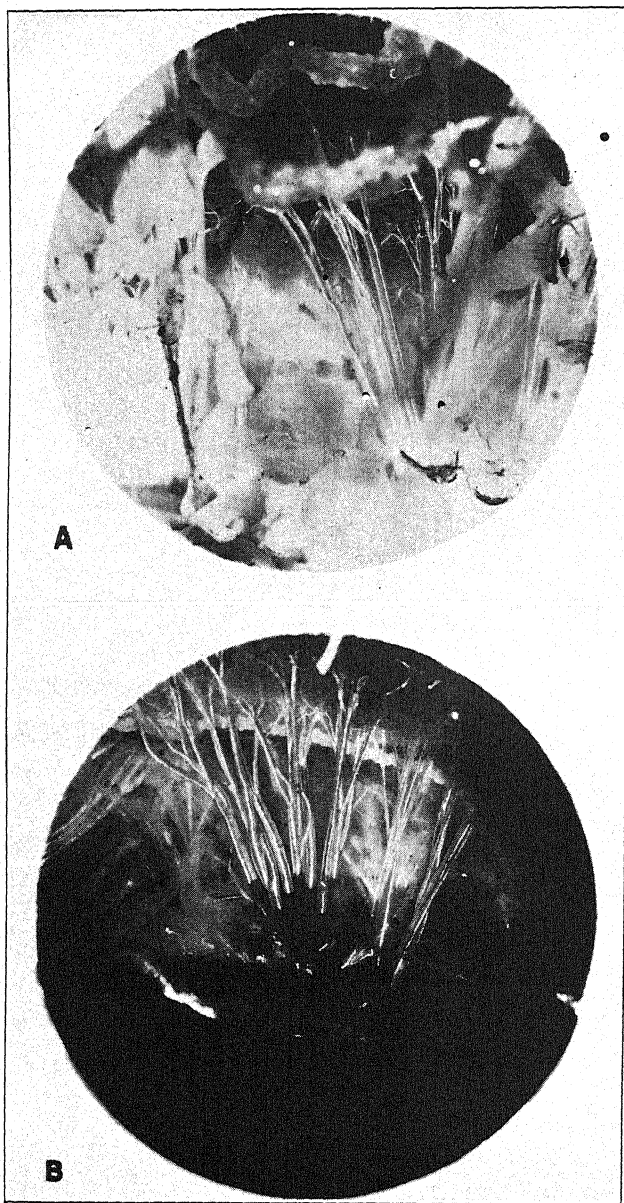


FIGURE 4. A. A larva that had previously been killed with KCN and then immersed in a soap and nicotine solution. It will be noted from the dissection that the tracheal tubes do not contain any of the spray solution. B. A larva that was immersed alive in a soap and nicotine solution prior to dissection. The tracheal tubes, it will be noted, are partly filled with spray solution.

the tracheal tubes showed that the contact angle was about  $85^\circ$ , and therefore the capillary forces tending to cause penetration must be exceedingly weak even in the case of such strong soap solutions. The solutions then must have been forced up the tracheal tubes by some other means, such as the respiratory movements of the insect. That this is the case is borne out by the fact that larvae previously killed with KCN and then submitted to dipping and dissection showed no penetration whatever (compare A and B, Fig. 4). Similar conclusions have been reached by McGovran (11) in regard to the penetration of sodium oleate solutions into the tracheae of the honey bee. While penetration of some of the tracheae was obtained, in every case that a suitable amount and kind of spreader was employed with aqueous nicotine solutions, yet in no case observed were all the tracheae penetrated. An examination of the upper portion of the tracheae near the point of attachment to the ventral nerve cord failed to reveal spray solution in visible amount. Apparently the spray solution enters and partially fills the tracheae, and a portion of the nicotine is volatilized within the tracheae and comes in contact with the nerves. McIndoo (12) has shown that the partial pressure of nicotine from dilute solutions is sufficient to kill through the vapor phase.

#### DISCUSSION

The experiments with the four solutions described above show that in these cases the surface forces between the spray solution and the insects are of paramount importance for their efficiency as contact insecticides. In spite of their high toxicity, nicotine solutions failed to give satisfactory kills unless a spreading agent was used which resulted in low surface tension and small angle of contact. The spreading coefficient appears to be a useful criterion of the ability of the solution to wet the insect and penetrate the tracheae.

#### SUMMARY

The rôle of surface forces in determining the efficiency of contact insecticides has been discussed.

Preliminary observations have shown that many spray solutions wet poorly and do not spread over the insect and form a film unless a suitable spreading agent is present. It was also found that aqueous spray solutions do not penetrate the tracheal system of the larva of the common tomato worm (*Phlegethontius quinquemaculata*) without a wetting agent. Among the wetting agents used, soaps were found to be the best. Even with the use of soap as a spreader, the solution did not penetrate the tracheal system of a tomato worm larva previously killed with KCN, indicating that respiratory movements or at least vital activity are necessary for penetration to take place. The angle of contact exhibited by soap solutions within the tracheae also indicates that the capillary forces involved can not account for penetration by this means alone.



The toxicity to *Aphis rumicis* of nicotine solutions containing no spreader, calcium caseinate, Penetrol, and sodium oleate has been determined and comparisons have been made between the toxicity and the spreading coefficients of these four solutions as determined by measurements of surface tension and angle of contact.

It was found in these four cases that the toxicity followed the same order as the spreading coefficient.

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# THE FUNGICIDAL ACTION OF SULPHUR. II. THE PRODUCTION OF HYDROGEN SULPHIDE BY SULPHURED LEAVES AND SPORES AND ITS TOXICITY TO SPORES<sup>1</sup>

S. E. A. MCCALLAN AND FRANK WILCOXON

In the first paper of this series (40) evidence was presented that the fungicidal action of sulphur cannot be ascribed to pentathionic acid. Preliminary results were included showing that hydrogen sulphide is highly toxic to the spores of the fungi tested, thus confirming the early findings of Pollacci (28) and the recent ones of Marsh (24). The present paper deals with the production of hydrogen sulphide from spores or leaves in contact with sulphur and includes a more detailed study of the toxicity of this gas to the spores of eight representative fungi.

## THE PRODUCTION OF HYDROGEN SULPHIDE FROM SULPHURED LEAVES AND SPORES

The fact that many kinds of living tissue reduce sulphur to hydrogen sulphide at ordinary temperatures has been recognized for half a century. Pollacci (28) in 1862 appears to have been the first to observe this property of living matter, since he reports that grape leaves treated with sulphur evolved hydrogen sulphide which he recognized by its odor and the blackening of lead acetate paper. Selmi (33) as well as Cugini (8) likewise observed the formation of hydrogen sulphide when sulphur is applied to certain fungi. In 1888 de Rey-Pailhade (31) stated that an alcoholic extract of yeast, on being mixed with sulphur, rapidly evolved hydrogen sulphide at ordinary temperatures. He showed that the substance producing the hydrogen sulphide exists in the living cell, and noted that the action is most rapid at a temperature of from 35° to 40° C., and is inhibited by previously heating the solution to 70° C. This property was not confined to yeast cells, but the same reaction was obtained from a variety of animal and vegetable tissues. De Rey-Pailhade (32) considered this reaction to be brought about by a specific enzyme to which he gave the name *Philothion*. During the next decade this reaction was studied by de Rey-Pailhade and other investigators, some of whom questioned its enzymatic nature (1, 2). A review by Pozzi-Escot (30) of the subject of the reducing enzymes presents the status of philothion in 1903. This author reports that philothion readily undergoes oxidation which destroys its power to reduce sulphur. He also observed that philothion was capable of reducing selenium, tellurium, arsenic, and phosphorus, forming the hydrides of these elements.

<sup>1</sup> Herman Frasch Foundation for Research in Agricultural Chemistry, Paper No. 18.

There is a reaction characteristic of compounds containing the  $-SH$  group, namely, the production of a violet color on treating with sodium nitroprusside and ammonia (37, p. 327). Several investigators had shown that a substance giving this test was widely distributed in plant and animal tissue (36). Heffter (14) in 1908 suggested that this  $-SH$  group, since it contains labile hydrogen, is the agent responsible for the reducing action of living cells.

In 1921 Hopkins (15) announced the isolation, from yeast and from liver, of a remarkable substance to which he gave the name *Glutathione*. This substance was later shown to be a tripeptid of glycine, glutamic acid and cysteine. It contains the  $-SH$  group, is readily oxidized under suitable conditions by atmospheric oxygen, and exhibits the property of reducing sulphur to hydrogen sulphide at ordinary temperatures. Hopkins considers this compound to be identical with the philothion of de Rey-Pailhade. Since its isolation, glutathione has been subjected to intensive study by Hopkins and his coworkers (25) and by Kendall and others (16), chiefly in connection with its relations to oxygen. The ability of glutathione to reduce sulphur is mentioned incidentally by Hopkins (15); the first detailed study is contained in a paper by Sluiter (35). She determined the yield of hydrogen sulphide obtained in two hours from a mixture of sulphur and glutathione of known concentration. The yield of hydrogen sulphide obtained from various animal tissues of known glutathione content, as found by Tunncliffe's method (39), was determined. No definite relation was apparent between the glutathione content of the different tissues and the hydrogen sulphide produced. This author also studied the effect of temperature on the reaction; tissues yielded hydrogen sulphide on boiling only when sulphur was added. It is easily demonstrated, however, that sulphur in contact with water yields hydrogen sulphide on heating. This reaction first becomes apparent at about  $65^{\circ} \text{C}$ . and is quite rapid at the boiling point of water. Lewis and Randall (17) have studied the hydrolysis of sulphur at its boiling point. Sluiter also found that the pH of the medium had a marked effect on the rate of production of hydrogen sulphide by animal tissues. An optimum rate was obtained at a pH of 7.8. This author concluded that hydrogen sulphide production from animal tissues on the addition of sulphur was a purely chemical process and not dependent on enzyme activity.

In 1929 Marsh (24) demonstrated that hydrogen sulphide is produced by the interaction of sulphur with several species of higher plants and fungi. The hydrogen sulphide was detected with lead acetate paper. Barker, in a progress report for 1929 (4), presents a list of 61 species of higher plants tested for their ability to form hydrogen sulphide when dusted with sulphur. Of these, 15 species gave a positive result as indicated by the darkening of lead acetate paper. A similar reaction was ob-

tained with 19 species of lower plants which included fungi, algae, and mosses, while five species of fungi gave a negative result. This author discussed the probable nature of the reaction and in a footnote suggested glutathione as the reducing factor.

Recently Liming and Young (18) also observed the evolution of hydrogen sulphide from sulphur in contact with leaves and spores.

It is thus apparent that there is a large amount of evidence that living tissues in contact with sulphur are able to bring about its reduction with the formation of hydrogen sulphide. But the views of different investigators as to the exact nature of the reaction are not in agreement, some holding that the reaction is independent of enzyme activity, others that it is dependent on reducing enzymes secreted by the living cell.

#### PRODUCTION OF HYDROGEN SULPHIDE BY LEAVES

##### *Qualitative Observations*

In order to demonstrate the evolution of hydrogen sulphide from leaves treated with sulphur, the following method was used. Small glass rings 18 mm. in diameter by 10 mm. deep were cemented to the surface of the leaf with a mixture of vaseline and beeswax. Ground roll sulphur was placed inside the cell to form a thin layer on the leaf surface. The cell was closed by a cover glass to which was fastened a little strip of moist lead acetate test paper. This paper indicates the presence of hydrogen sulphide by its blackening due to the formation of lead sulphide. The cover glass was secured to the ring by the vaseline-beeswax mixture thus forming an air-tight chamber. These experiments were performed on plants growing out-of-doors during the month of May when sunny weather and a temperature of about 85° F. prevailed during the middle portion of the day. Similar cells containing no sulphur and also cells containing sulphur and cemented to glass slides served as controls. Twenty-six species of plants representing 16 families were tested, and in every case hydrogen sulphide was produced. However, there was a marked variation in the rate of production by different species. These results appear in Table I.

In the majority of cases the evolution of hydrogen sulphide was more rapid from the lower than the upper surface of the leaf. This is perhaps correlated with the more frequent occurrence of stomata on the lower surface. The property of rapidly producing hydrogen sulphide appears to be distributed uniformly among the members of the Rosaceae. In some species the evolution of hydrogen sulphide was very noticeable within an hour. The control cells showed no production of hydrogen sulphide. In order to confirm the formation of hydrogen sulphide a drop of dilute sodium hydroxide solution was substituted for the lead acetate paper. After several hours this drop was transferred to a glass slide and the sulphide converted

to methylene blue by treatment with p-aminodimethylaniline hydrochloride and ferric chloride (37, p. 327).

These results confirm, in general, the observations of Barker (4) except that positive results were obtained in all cases in the present investigations. It is apparent that this property of producing hydrogen sulphide from sulphur is widespread among the higher plants.

TABLE I

THE PRODUCTION OF HYDROGEN SULPHIDE FROM SULPHUR IN CONTACT WITH THE LEAVES OF VARIOUS PLANTS

Species	Upper surface		Lower surface	
	4 hours	24 hours	4 hours	24 hours
<i>Dahlia pinnata</i> Cav.....	x*	xx	x	xx
<i>Lactuca sativa</i> L.....	xx	xx	xxx	xxxx
<i>Cucumis sativus</i> L.....	x	xx	xxx	xxxx
<i>Lycopersicum esculentum</i> Mill.....	x	xx	xxx	xxxx
<i>Nicotiana tabacum</i> L.....	x	xx	x	xx
<i>Solanum tuberosum</i> L.....	o	o	o	xxx
<i>Coleus Blumei</i> Benth.....	o	o	o	xx
<i>Syringa vulgaris</i> L.....	o	xx	o	xx
<i>Apium graveolens</i> L.....	o	x	x	xxx
<i>Vitis Labruscana</i> Bailey.....	o	xx	x	xxx
<i>Parthenocissus quinquefolia</i> Planch.....	x	xx	x	xxx
<i>Phaseolus vulgaris</i> L.....	o	o	x	xx
<i>Pisum sativum</i> L.....	xxxx	xxxx	xxxx	xxxx
<i>Fragaria chiloensis</i> Duchesne.....	xx	xxx	xxxx	xxxx
<i>Prunus avium</i> L.....	xx	xxx	xx	xxxx
<i>Prunus persica</i> (L.) Stokes.....	xx	xxx	xxx	xxxx
<i>Pyrus malus</i> L.....	xx	xxx	xxxx	xxxx
<i>Rosa rugosa</i> Thunb.....	xx	xxx	xxxx	xxxx
<i>Rubus idaeus</i> L.....	x	xx	xxxx	xxxx
<i>Ribes sativum</i> Syme.....	o	xx	x	xxxx
<i>Brassica oleracea</i> L.....	xxx	xxxx	xxx	xxxx
<i>Beta vulgaris</i> L.....	xxx	xxx	xxxx	xxxx
<i>Rheum Rhaiponticum</i> L.....	o	o	xx	xxx
<i>Tulipa Gesneriana</i> L.....	x	xx	x	xx
<i>Zea mays</i> L.....	x	xx	x	xxx
<i>Ginkgo biloba</i> L.....	o	x	xxx	xxxx
Controls { Cell on leaf no sulphur.....	o	o	o	o
{ Cell with sulphur on glass slide.....	o	o	o	o

\* xxxx Very heavy test, xxx Heavy test, xx Medium test, x Light test, o No test.

### Quantitative Experiments

The experiments described in the preceding section only furnished qualitative data and it seemed desirable to attempt a quantitative estimation of the amount of hydrogen sulphide produced in a given time from a known area of leaf surface. A potted strawberry plant thoroughly dusted with finely divided sulphur was placed in an anatomical jar with a tightly fitting cover furnished with an inlet and outlet tube. The jar was immersed in a water bath maintained at constant temperature. By means of a water

through dilute sodium hydroxide solution. The hydrogen sulphide evolved in a given period of time was absorbed by the sodium hydroxide solution and determined by conversion to methylene blue (3). The total leaf area, including the upper and lower surfaces, was determined. This permitted the calculation of the rate of production of hydrogen sulphide per hour per unit area of leaf surface. These results are shown in Table II.

TABLE II  
THE RATE OF EVOLUTION OF HYDROGEN SULPHIDE FROM A SULPHUR  
DUSTED STRAWBERRY PLANT AT 35° C.

Experiment	Area leaf surface sq. cm.	Time, hours	Mg. H <sub>2</sub> S per plant per hour	Mg. H <sub>2</sub> S per hour per sq. dm. leaf surface
1	460	0.5	0.0174	0.0038
		13.5	0.0076	0.0016
		16.8	0.0087	0.0019
2	228	1.0	0.0041	0.0018
		3.0	0.0038	0.0017
		5.0	0.0051	0.0022
		25.0	0.0039	0.0017
Average rate milligrams H <sub>2</sub> S per hour per sq. dm. leaf surface.....				0.0021
Total milligrams H <sub>2</sub> S evolved per 12 hours per sq. dm. leaf surface.....				0.0252

It will be observed that the rate of evolution of hydrogen sulphide from the plant over a period of at least 25 hours is fairly constant. After this time the plants began to show symptoms of injury and the experiments were discontinued.

#### PRODUCTION OF HYDROGEN SULPHIDE BY FUNGUS SPORES

##### *Qualitative Observations*

The ability of fungus spores to evolve hydrogen sulphide in the presence of sulphur was tested in the following manner. About five grams of a sulphur paste made from ground roll sulphur and containing 50 per cent of water was introduced into small wide-mouthed bottles of 110 cc. capacity. An aqueous suspension of the spores to be tested was thoroughly mixed with the sulphur paste and the bottle closed with a rubber stopper from which was suspended a piece of moist lead acetate paper. The time required to produce an appreciable darkening of the lead acetate paper at room temperature varied from about ten minutes to several hours depending on the species. Spores from the following species were tested and all gave positive results:

*Botrytis allii* Munn.—conidia

*Diaporthe umbrina* Jenk.—conidia

*Botrytis* sp. (*cinerea* type)—conidia

*Endothia parasitica* (Murr.) And. &

And.—conidia	<i>Venturia inaequalis</i> (Cke.) Wint.—
<i>Glomerella cingulata</i> (St.) Sp. & von	conidia
S.—conidia	<i>Tilletia laevis</i> Kühn—chlamydo-
<i>Macrosporium sarcinaeforme</i> Cav.—	spores
conidia	<i>Tilletia tritici</i> (Bjerk.) Wint.—chla-
<i>Neurospora sitophila</i> (Mont.) Shear	mydosporos
& Dodge—conidia	<i>Ustilago zeae</i> (Beck.) Unger—chla-
<i>Penicillium</i> sp.—conidia	mydosporos
<i>Pestalotia macrotricha</i> Klebahn.—	<i>Puccinia antirrhini</i> Diet. & Holw.—
conidia	uredosporos
<i>Pestalotia stellata</i> B. & C.—conidia	<i>Uromyces caryophyllinus</i> (Schr.)
<i>Sclerotinia americana</i> (Worm.) Nort.	• Wint.—uredosporos
& Ezek.—conidia	

Controls consisting of similar bottles containing sulphur paste as well as bottles containing spores without sulphur did not produce hydrogen sulphide under these conditions.

Tests for hydrogen sulphide production were also made with macerated sporophores of *Agaricus campestris* L., *Armillaria mellea* (Fr.) Quel., and *Pleurotus ostreatus* Fr. These three species all gave a positive test in a few minutes at 30° C. Freshly expressed filtered juice from sporophores of *Pleurotus ostreatus* when added to sulphur paste also rapidly produced hydrogen sulphide. Numerous attempts have been made to demonstrate the production of hydrogen sulphide from filtrates from spore suspensions and from the filtered water in which spores had germinated, but without success.

### Quantitative Experiments

It frequently happens in dealing with a chemical reaction, the mechanism of which is unknown, that valuable information can be obtained by a study of the kinetics of the reaction as affected by temperature, concentration of reagents, and hydrogen ion concentration of the medium. In addition, it seemed desirable to determine the yield of hydrogen sulphide obtained from different species of fungous spores under definite conditions. Although the ability of living tissues in contact with sulphur to evolve hydrogen sulphide has been known for many years, apparently the only previous study along these lines is the recent one of Sluiter (35).

*Methods.* Eight representative phytopathogenic fungi, the spores of which exhibit varying degrees of sulphur sensitivity, as will be shown later, were selected for the detailed study of hydrogen sulphide production and hydrogen sulphide toxicity of this investigation. Four of these species, *Sclerotinia americana* (Worm.) Nort. & Ezek., *Botrytis* sp., of the *cinerea* type, *Macrosporium sarcinaeforme* Cav., and *Uromyces caryophyllinus* (Schr.) Wint. were the same as those studied and discussed in the first

paper of this series (40). The four additional species were *Glomerella cingulata* (St.) Sp. and von S., *Pestalotia stellata* B. & C., *Venturia inaequalis* (Cke.) Wint., and *Puccinia antirrhini* Diet. & Holw. The culture of *Glomerella cingulata* was obtained from Dr. H. W. Anderson of the University of Illinois. The *Pestalotia stellata* was isolated from *Ilex opaca* Ait., at Sea Girt, New Jersey, by Dr. R. P. White. These two species sporulate abundantly in potato dextrose agar and the conidia thus obtained germinate readily in redistilled water at 20°–23° C., the temperature employed. Conidia of *Venturia inaequalis* were obtained directly from the fruit of naturally infected McIntosh apples. Doran (10) has shown that *Venturia inaequalis* conidia rapidly lose their power of germinating with age. This fact probably explains the somewhat low percentage of germination obtained, as these conidia were studied in the late autumn. Germination tests were made at 14.5° C. Uredospores of *Puccinia antirrhini* were obtained from naturally infected snapdragon plants growing out-of-doors. These spores did not exhibit the extremely narrow optimum temperature for germination reported by Doran (9), for in redistilled water but little difference was observed from 5° to 20° C. The temperature 14.5° C. was selected for germination tests.

The actual weight of spore material available for a study of hydrogen sulphide production is relatively small, and the hydrogen sulphide evolved represents only a small fraction of the weight of the spores, therefore it was necessary to use exceedingly sensitive methods for its determination.

Two methods are available for the determination of small amounts of hydrogen sulphide; namely, the method described by Almy (3) in which hydrogen sulphide is determined as methylene blue, and the method depending on the coloration imparted to lead acetate paper which was thoroughly studied by McBride and Edwards (19). The first of these, although more accurate, is only sensitive to about 0.01 milligrams of hydrogen sulphide, whereas by the use of lead acetate paper amounts as small as 0.001 milligrams could be detected and determined approximately. The second method was found more suitable and the procedure adopted was the same as that outlined above. Pieces of Whatman No. 2 filter paper were dipped in a 10 per cent solution of Horne's basic lead acetate and cut to form strips 3.0 by 0.6 cm. tapered at one end. These strips while still moist were suspended in the bottles of sulphur paste and spores. The darkening imparted to the test papers by 20 parts per million or 0.0033 milligrams of hydrogen sulphide was adopted as standard, and from the time required to produce this coloration the rate of evolution of hydrogen sulphide in milligrams per hour could be calculated. The method was standardized by introducing a sufficient amount of hydrogen sulphide into a similar bottle to impart the same degree of darkening to the paper. The coloration corresponding to 20 parts per million was adopted because it was found most



suitable for comparisons. Between determinations a current of moist air was passed through the bottles in order to sweep out the hydrogen sulphide and prevent accumulation of the reaction products.

In these experiments a definite volume of spore suspension was used in each test. The number of spores in a unit volume of suspension was determined by means of a Fuchs-Rosenthal counting cell. In order to determine the weight of spores used, it was necessary to estimate their volume and specific gravity. The volume was estimated from linear measurements of the spores made by means of an ocular micrometer. The specific gravity was obtained by the application of Stokes' law of falling bodies to rate of

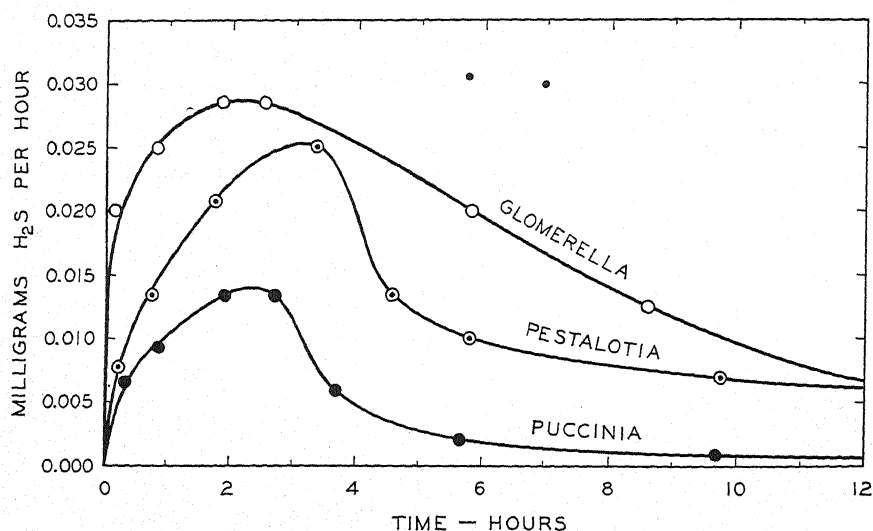


FIGURE 1. The rate of production of hydrogen sulphide from sulphur by 4,000,000 conidia of *Glomerella cingulata*, 240,000,000 conidia of *Pestalotia stellata*, and 6,000,000 uredospores of *Puccinia antirrhini*.

fall of spores in water. The rate of fall of the spores was determined in a counting cell placed on the stage of a horizontal microscope as described by Buller (6). Assuming the applicability of Stokes' law to this case, the specific gravity of the spores of the eight fungi used were found to vary from 1.07 to 1.14, depending on the species. Thus, knowing the number of spores, their volume, and specific gravity together with the rate of evolution of hydrogen sulphide, it was possible to calculate the ratio between the weight of hydrogen sulphide evolved in a given time and the weight of spores.

*Rate of evolution.* When sulphur is brought into contact with fungous spores at about 30° C., evolution of hydrogen sulphide commences at a relatively slow rate which progressively increases to a well defined maximum, reached after about two to four hours, depending on the conditions.

Thereafter the rate falls exponentially and reaches a very low value at 48 hours.

Rate curves for hydrogen sulphide production by the spores of the eight fungi employed have been obtained. Representative curves for three different species are shown in Figure 1.

It will be observed that the curves are not symmetrical. Reaction velocity curves of this type are characteristic of processes taking place in two stages and have been discussed by Osterhout (27, p. 57). However, such curves do not give information as to the exact nature of the two processes concerned.

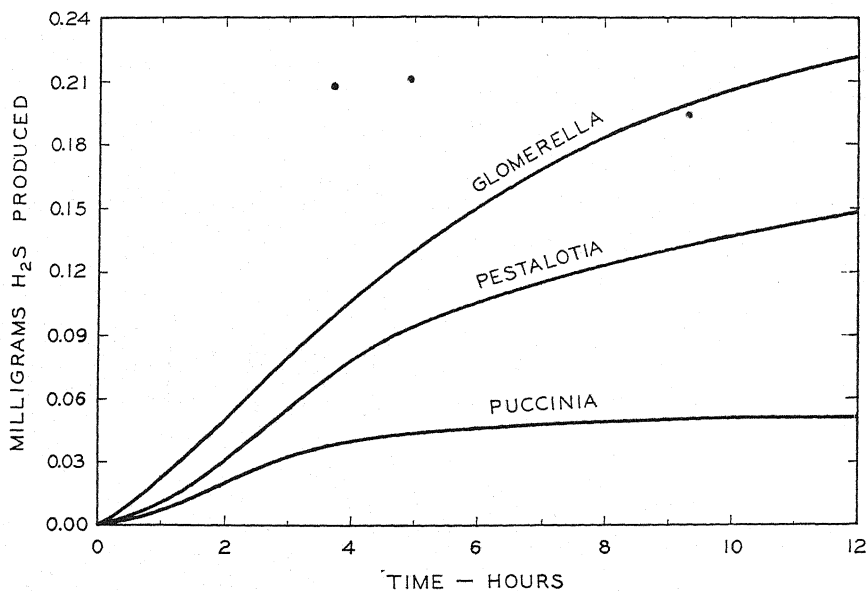


FIGURE 2. The total production of hydrogen sulphide from sulphur by spores of *Glomerella cingulata*, *Pestalotia stellata*, and *Puccinia antirrhini* as derived from Figure 1.

The total production of hydrogen sulphide up to any given time may be obtained from the rate curve by measuring the area under the curve up to that time. Such total production curves plotted against time as obtained from Figure 1 are shown in Figure 2. The average total production of hydrogen sulphide over a period of 12 hours for the eight fungi studied, as obtained by this method, are given in Table III.

It will be observed that the amount of hydrogen sulphide evolved varies greatly with different species, and is especially large for the conidia of *Glomerella cingulata* and *Sclerotinia americana*. These results are the average of a number of experiments and it was found that the amount produced by a given species is somewhat variable in different experiments. There appears to be a relationship between the specific gravity of the

spores and the amount of hydrogen sulphide evolved, the production being greater in the case of the lighter spores.

TABLE III

THE TOTAL PRODUCTION OF HYDROGEN SULPHIDE FROM SULPHUR OVER A PERIOD OF 12 HOURS AT 30° C. BY THE SPORES OF EIGHT FUNGI

Species	Specific gravity of spores	Total mg. H <sub>2</sub> S per mg. spores	H <sub>2</sub> S evolved as per cent weight of spores
<i>Glomerella cingulata</i> .....	1.08	0.098	9.8
<i>Sclerotinia americana</i> .....	1.07	0.051	5.1
<i>Botrytis</i> sp. (cinerea type).....	1.12	0.0029	0.29
<i>Venturia inaequalis</i> .....	1.12	0.0027	0.27
<i>Pestalotia stellata</i> .....	1.11	0.0024	0.24
<i>Uromyces caryophyllinus</i> .....	1.13	0.0020	0.20
<i>Puccinia antirrhini</i> .....	1.14	0.0019	0.19
<i>Macrosporium sarcinaeforme</i> .....	1.14	0.0014	0.14

The amount of hydrogen sulphide evolved is directly proportional to the number of spores as is illustrated in Table IV. A similar relation has been shown in the case of alcoholic fermentation by yeasts (13, p. 50).

TABLE IV

THE RELATION BETWEEN THE NUMBER OF SPORES AND THE AMOUNT OF HYDROGEN SULPHIDE EVOLVED FOR THREE SPECIES OF FUNGI

Species	Number of spores	Total mg. H <sub>2</sub> S produced by spores, 12 hrs. at 30° C.	Total mg. H <sub>2</sub> S per mg. spores
<i>Glomerella cingulata</i>	2,675,000	0.069	0.093
	5,250,000	0.151	0.102
<i>Sclerotinia americana</i>	2,000,000	0.086	0.056
	4,000,000	0.150	0.049
	4,500,000	0.158	0.046
	5,000,000	0.231	0.060
<i>Puccinia antirrhini</i>	715,000	0.0125	0.0022
	1,430,000	0.0192	0.0019
	2,145,000	0.0335	0.0024

*Effect of temperature.* Previous qualitative observations had indicated that the reaction between spores and sulphur is sensitive to the influence of temperature. Moreover, it was expected that the effect of temperature on the reaction would indicate whether or not the reaction is promoted by an enzyme, since most enzyme reactions are sensitive to temperature.

Accordingly, curves for the rate of production of hydrogen sulphide by 4,500,000 conidia of *Sclerotinia americana* were obtained at temperatures of 0°, 10°, 12.5°, 18°, 25°, 30°, 35°, 45°, 55°, and 65° C. The results for six

different temperatures are shown in Figure 3, and the total amounts produced in 12 hours for the various temperatures in Figure 4.

In the above curves, the spores were added to the sulphur and the mixture maintained at the desired temperature throughout the course of the experiment. It was also considered of interest to determine the effect of preheating the spores on their subsequent total production of hydrogen sulphide at 30° C. The results obtained for 3,000,000 conidia of *Sclerotinia*

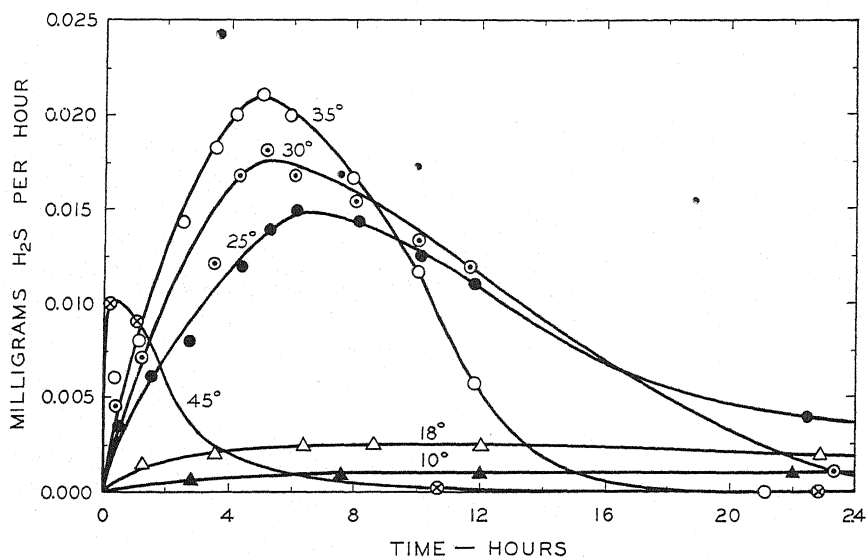


FIGURE 3. The rate of production of hydrogen sulphide from sulphur by 4,500,000 conidia of *Sclerotinia americana* at various temperatures.

*americana*, previously heated for ten minutes at 30°, 45°, 55°, 65°, and 75° C., is given in Table V.

TABLE V

THE EFFECT OF HEATING CONIDIA OF *SCLEROTINIA AMERICANA* FOR 10 MINUTES AT VARIOUS TEMPERATURES ON THE SUBSEQUENT TOTAL HYDROGEN SULPHIDE PRODUCTION FROM SULPHUR IN 12 HOURS AT 30° C.

Temperature ° C.	Total mg. H <sub>2</sub> S per 3,000,000 spores	Total mg. H <sub>2</sub> S per mg. spores
30	0.101	0.046
45	0.074	0.033
55	0.000	0.000
65	0.000	0.000
75	0.000	0.000

From Figure 3 it can be seen that at high temperatures the maximum rate of evolution of hydrogen sulphide is quickly reached and then falls off sharply. At low temperatures there is no well defined maximum and the

rate is slow throughout. Figure 4 shows that the greatest evolution of hydrogen sulphide, over a period of 12 hours, occurs at about 35° C. Previous heating of the spores for ten minutes at 55° C. appears to destroy completely their power of producing hydrogen sulphide from sulphur as is shown in Table V.

The effect of temperature on this reaction, as shown by these curves, is strongly indicative of enzyme action. Certain previous investigators (1, 2, 4, 35) have drawn the conclusion that the reaction is not enzymatic in nature. They appear to have overlooked the fact that sulphur itself in the

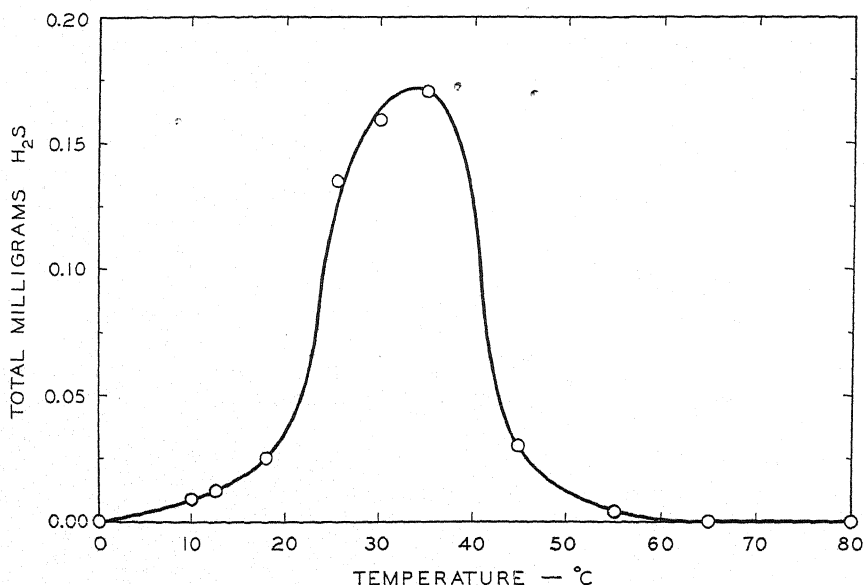


FIGURE 4. The total production of hydrogen sulphide from sulphur by 4,500,000 conidia of *Sclerotinia americana* after 12 hours at various temperatures.

absence of spores will yield hydrogen sulphide if heated in the presence of moisture at 65° C. and above.

*Effect of pH.* It has been found by Sluiter (35) that the rapidity with which animal tissues are able to reduce sulphur is largely dependent on the acidity of the medium and exhibits a sharp maximum at a pH of 7.8. It was anticipated, therefore, that the ability of fungous spores to produce hydrogen sulphide when in contact with sulphur would also be affected by the reaction of the medium. Experiments were performed with conidia of *Sclerotinia americana*, *Macrosporium sarcinaeforme*, *Pestalotia stellata*, *Botrytis allii*, and *Endothia parasitica* and chlamydospores of *Ustilago zeae* using Clark and Lubs buffers (7) at half the usual concentration. It was found that within the pH range of about 4.0 to 8.0 the rate of evolution is

independent of the H ion concentration and no well defined maximum was to be observed. This perhaps indicates that the reaction takes place within the spore and hence will not be readily affected by the external pH.

#### *Nature of the Reaction*

The original view of de Rey-Pailhade (32) that this reaction is enzymatic in nature has been contested by Abelous and Ribaut (1, 2) and Sluiter (35) on the grounds that the amounts of hydrogen sulphide produced were strictly limited and that its production was observed at temperatures destructive to most enzymes. Barker (4) also considered the reaction one of a purely chemical nature. The fact that the production of hydrogen sulphide is somewhat limited does not necessarily indicate that the reaction is not enzymatic, for the product hydrogen sulphide is known to inhibit the activity of certain enzymes (34, p. 689). As has been pointed out previously, sulphur at higher temperatures is hydrolyzed with the production of hydrogen sulphide and this fact does not appear to have been given due consideration by these authors. The quantitative temperature relations as shown in Figures 3 and 4, and Table V appear, however, to leave but little doubt that an enzyme is concerned in the reaction.

An important question which presents itself in connection with this reaction is that of its location. Does the reaction take place on the sulphur particle or on or within the spore? A number of experiments were performed to obtain information on this point.

Collodion sacs were prepared according to the formula, 25 cc. absolute ethyl alcohol, 75 cc. ether, and 2.5 grams soluble negative cotton (21). A heavy suspension of spores of *Sclerotinia americana* was placed in the sac and the sac suspended in a small vial containing sulphur paste in such a manner that the sulphur paste surrounded the spore suspension. Both sac and vial were closed with rubber stoppers from which were hung strips of lead acetate test paper as illustrated in Figure 5. After standing overnight at 25°C. a noticeable darkening of the test paper within the sac was apparent, while the test paper over the sulphur paste remained white. This experiment was repeated several times with the same results, thus demonstrating that the hydrogen sulphide is formed on or in the spores and not on the sulphur particles. Some substance, therefore, must have diffused from the particles to the spores. Such substances might be the acids associated with sulphur or sulphur itself. It was found, however, that water extracts from sulphur do not produce hydrogen sulphide when spores are immersed in them. Solutions containing 0.5 per cent sulphuric acid, sulphurous acid, and sodium bisulphate failed to produce any hydrogen sulphide when in contact with spores, while sodium thiosulphate and potassium pentathionate gave slight traces, but far less than was evolved from the same amount of sulphur itself.

That elementary sulphur exerts an appreciable vapor pressure and can bring about chemical action at a distance, has been shown by Zenghelis (41) and by Tucker (38). The former noted the blackening of silver foil sus-

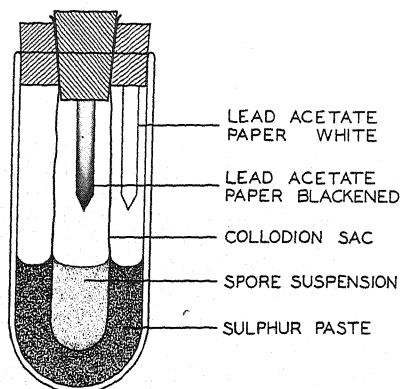


FIGURE 5. The production of hydrogen sulphide by *Sclerotinia* spores separated from sulphur by a collodion membrane. Note that the evolution of hydrogen sulphide takes place on the spore side of the membrane and not on the sulphur side.

pended above sulphur but not in contact with it in a closed vessel and the latter made observations on the rate of sublimation of sulphur at ordinary temperatures. Data on the vapor pressure of sulphur are given in the International Critical Tables (3: 201) for temperatures down to  $50^{\circ}$  C. where the vapor pressure is 0.0002 mm. Extrapolation of the vapor pressure curve to  $25^{\circ}$  C. would indicate a pressure of about 0.00002 mm. at that temperature. The writers have confirmed the fact that sulphur will corrode a bright silver wire suspended above it in a closed bottle. The following experiment, illustrated in Figure 6, was performed in order to determine whether or not sulphur can react with spores across an air space to produce hydrogen sulphide. A piece of glass tubing 5 cm.

long and 0.5 cm. bore was cemented to a glass slide. Two pieces of glass rod inserted at either end of the tubing made a chamber of adjustable size. A single crystal of carefully washed rhombic sulphur was placed at one side of the chamber. On the end of the glass rod, which formed the other side of the chamber, was placed a coating of yeast cells. Between the sulphur crystal and the yeast cells was suspended a fragment of filter paper impregnated with lead acetate solution. The distance between the sulphur crystal and the yeast cells was from 3 to 4 mm. After 12 hours a darkening of the lead acetate paper was apparent. This experiment was repeated several times with similar results. Thus it appears that actual contact between sulphur and spores is not essential for the production of hydrogen sulphide. The simplest explanation for the results of these experiments is that sulphur vapor continuously evolved in small amounts from the sulphur particles is responsible for the action observed.

The wide distribution in plant and animal tissues of substances containing the mercaptan or -SH groups has been mentioned previously. The writers have been able to demonstrate the presence of such a compound in the conidia of *Sclerotinia americana*. Five grams of these spores were ground in a mortar with 10 cc. of 10 per cent trichloroacetic acid and quartz sand. The filtered extract gave a distinct test for the -SH

group with ammonia and sodium nitroprusside. The ease with which such substances reduce sulphur to hydrogen sulphide is well known (23). It seems highly probable, therefore, that such compounds are involved in the present reaction. Evidence has been submitted, however, that the reaction is dependent on an enzyme. It is suggested, therefore, that both these factors, namely, an enzyme and a -SH compound, are necessary for the production of hydrogen sulphide as it occurs in the living spore in contact with sulphur. However, this explanation is not entirely sufficient in all cases, for at least the spores of certain species of fungi evolve far more hydrogen sulphide than could be accounted for by any possible content of preformed -SH compound.

#### THE TOXICITY OF HYDROGEN SULPHIDE TO FUNGUS SPORES

It has been shown that sulphur in contact with leaves or fungous spores evolves hydrogen sulphide in very appreciable amounts and at a rate which varies with the conditions of the experiment. The bearing of this reaction on the fungicidal action of sulphur necessitates an accurate study of the toxicity of hydrogen sulphide to fungous spores. Preliminary observations on this topic were given in the first paper of this series (40) and the results of a more detailed study will now be presented.

Pollacci (28) in 1862 appears to have been the first to note that hydrogen sulphide exhibits toxicity towards fungi, since he demonstrated that this gas inhibits the growth of the "oidium" on the grape.

Foreman (12) reported that the germination of spores of *Botrytis cinerea* was not inhibited in saturated solutions of hydrogen sulphide. However, Barker, Gimingham, and Wiltshire (5) found that a 0.002 per cent solution of hydrogen sulphide inhibited the germination of spores of *Sclerotinia fructigena* when the experiment was performed in an enclosed space, although similar experiments in a vessel of a larger volume resulted in excellent germination.

A more extensive investigation was undertaken by Marsh (24) in 1929. This author exposed spores of *Botrytis cinerea* and *Monilia fructigena* to a 1 to 40,000 mixture of hydrogen sulphide in air in a four-liter bell jar, and obtained a marked reduction in germination. Several other species were tested using various concentrations of the gas and were also found sensi-

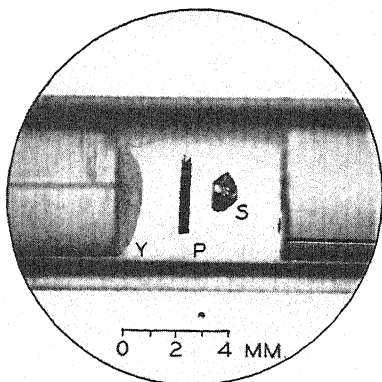


FIGURE 6. The action of sulphur (S) across an air space on yeast spores (Y), as indicated by the blackening of lead acetate paper (P).



tive. Additional experiments were performed in which a stream of hydrogen sulphide gas of known concentration was allowed to flow through a cell containing spores of various species. In this case also toxicity was demonstrated. Marsh showed further that hydrogen sulphide evolved from a mixture of spores and sulphur reduced the germ tube<sup>9</sup> length of other spores suspended several millimeters above and with a cellulose filter intervening.

#### EXPERIMENTAL METHODS

The determination of toxicity in this investigation was made by means of the spore germination tests previously described (20, 40). In working with hydrogen sulphide, however, certain properties of this substance (26) necessitate precautions which need not be considered when dealing with solutions of non-volatile substances such as copper sulphate, sulphuric acid, and pentathionic acid. Hydrogen sulphide is soluble in water to an extent which depends on its partial pressure above the solution. To any given concentration in the liquid there is a corresponding definite partial pressure in the vapor above. Unless this partial pressure is maintained constant the hydrogen sulphide rapidly escapes from the liquid. Therefore, in performing toxicity experiments with hydrogen sulphide it is essential to work in a closed system and to consider the relative volumes of liquid and vapor phase. Neglect of this property has led certain previous investigators to underestimate the toxicity of hydrogen sulphide. In addition to this, dilute mixtures of hydrogen sulphide with air as well as its solutions in water are relatively unstable and the hydrogen sulphide is rapidly oxidized, forming largely sulphur with small amounts of sulphuric acid (29).

In these experiments the usual moist chambers were fitted with an inlet tube through which measured volumes of hydrogen sulphide-air mixtures were admitted from a gas burette. The chambers were sealed with a ring of moistened filter paper. The concentration of hydrogen sulphide in the drops of spore suspension was calculated from Henry's law which has been shown to hold closely for this gas (22). The hydrogen sulphide admitted into the chambers distributed itself between the liquid and vapor phase in a constant ratio. Hence, with the volume of the moist chamber and that of the liquid known it is possible to calculate the concentration of hydrogen sulphide in each.

The loss of hydrogen sulphide from these chambers by oxidation, adsorption, and diffusion was determined by analyses at definite intervals. The method of Almy (3) was used. It was found that in the case of the most concentrated mixture used 50 per cent of the hydrogen sulphide had disappeared after three hours, while in the case of the dilute mixtures the loss was negligible. Studies on the rate of disappearance of

hydrogen sulphide in hermetically sealed bottles indicated that the major part of the loss is due to oxidation of the hydrogen sulphide.

The hydrogen sulphide was obtained from two sources, the commercial tank gas and that from the usual Kipp generator. The gas from the latter contained in addition to hydrogen sulphide, hydrogen, air, and about 50 parts per million of arsine. The gases from these two sources exhibited identical toxicity when compared on the basis of their actual hydrogen sulphide content. Separate experiments showed that arsine is approximately as toxic as hydrogen sulphide, but since it was present in such small amounts in the Kipp generator gas it had no toxic effect.

The fungi employed for the study of  $H_2S$  toxicity were the eight discussed in detail above, namely: *Venturia inaequalis*, *Uromyces caryophyllinus*, *Puccinia antirrhini*, *Sclerotinia americana*, *Macrosporium sarcinaeforme*, *Pestalotia stellata*, *Glomerella cingulata*, and *Botrytis* sp. (*cinerea* type). In all cases the spores were examined for germination after 22 hours. The tests with *Venturia* and *Puccinia* were run at  $14^\circ$  to  $15^\circ$  C., while the temperature for the other fungi was  $22^\circ$  to  $23^\circ$  C. All experiments were performed in duplicate, about 1000 spores being counted, and usually the results of two or more of these duplicate experiments were averaged to locate a single point on the toxicity curve (Fig. 7).

#### THE TOXICITY OF HYDROGEN SULPHIDE

The toxicity of hydrogen sulphide to the spores of these eight fungi was determined by the methods described above and the results are presented in Table VI and Figure 7.

It was found that the hydrogen sulphide is somewhat more toxic than the preliminary experiments (40) indicated. In Table VI and Figure 7 initial concentrations have been given. During the course of each experiment, as explained previously, the actual concentration within the moist chambers was continually decreasing. If this had not been the case, the gas would probably have exhibited even more toxicity than these figures indicate. It will be observed that there is a marked difference in sensitivity to hydrogen sulphide among these fungi. In the case of *Venturia*, the most sensitive, and *Botrytis*, the most resistant, the concentrations causing 50 per cent reduction in germination are in the ratio of 1 to .256. In some cases stimulation was evident at the lower concentrations. This was especially marked with *Pestalotia stellata*. The sigmoid curves obtained resembled in general those of sulphuric and pentathionic acid discussed in the first paper of this series (40). However, their lack of symmetry may perhaps be due to the fact that at the higher concentrations the rate of disappearance of hydrogen sulphide is greater than at the lower concentrations.

Since hydrogen sulphide undergoes a gradual oxidation during the course of the toxicity experiment it appeared possible that its toxicity

TABLE VI

THE TOXICITY OF HYDROGEN SULPHIDE TO THE UREDOSPORES OF *UROMYCES CARYOPHYLLINUS* AND *PUCCINIA ANTIRRHINI* AND TO THE CONIDIA OF *VENTURIA INAEQUALIS*, *SCLEROTINIA AMERICANA*, *MACROSPORIUM SARCINAEFORME*, *PESTALOTIA STELLATA*, *GLOMERELLA CINGULATA*, AND *BOTRYTIS SP. (CINEREA TYPE)*

Concentration: milligrams per liter solution	<i>Venturia inaequalis</i>		<i>Uromyces caryophyllinus</i>		<i>Puccinia antirrhini</i>		<i>Sclerotinia americana</i>		<i>Macrosporium sarcinaeforme</i>		<i>Pestalotia stellata</i>		<i>Glomerella cingulata</i>		<i>Botrytis sp. (cinerea type)</i>	
	Per cent germi- nation	Germ- tube length ( $\mu$ )	Per cent germi- nation	Germ- tube length ( $\mu$ )	Per cent germi- nation	Germ- tube length ( $\mu$ )	Per cent germi- nation	Germ- tube length ( $\mu$ )	Per cent germi- nation	Germ- tube length ( $\mu$ )	Per cent germi- nation	Germ- tube length ( $\mu$ )	Per cent germi- nation	Germ- tube length ( $\mu$ )	Per cent germi- nation	Germ- tube length ( $\mu$ )
Control	61.9	100	84.2	400	92.1	500	98.0	300	98.5	400	90.0	250	98.5	160	99.2	200
0.006	50.4	50	66.8	75	84.9	350	98.5	400	—	—	—	—	—	—	—	—
0.02	27.0	30	—	—	—	—	—	—	—	—	—	—	—	—	—	—
0.06	2.9	10	6.4	30	65.9	150	93.8	150	94.3	225	96.0	275	—	—	—	—
0.20	—	—	—	—	7.4	25	60.0	45	72.8	90	75.2	90	—	—	—	—
0.60	0	0	0.3	10	0.3	10	7.5	20	31.2	50	34.1	30	90.0	220	99.4	230
2.00	—	—	—	—	—	—	0.4	10	1.2	15	2.8	15	84.8	150	95.0	200
6.00	0	0	0	0	0	0	—	—	—	—	—	—	7.5	70	58.7	120
20.00	—	—	—	—	—	—	—	—	0	0	0	0	0.2	20	3.8	50
60.00	—	—	—	—	—	—	—	—	—	—	0	0	0	0	0	0

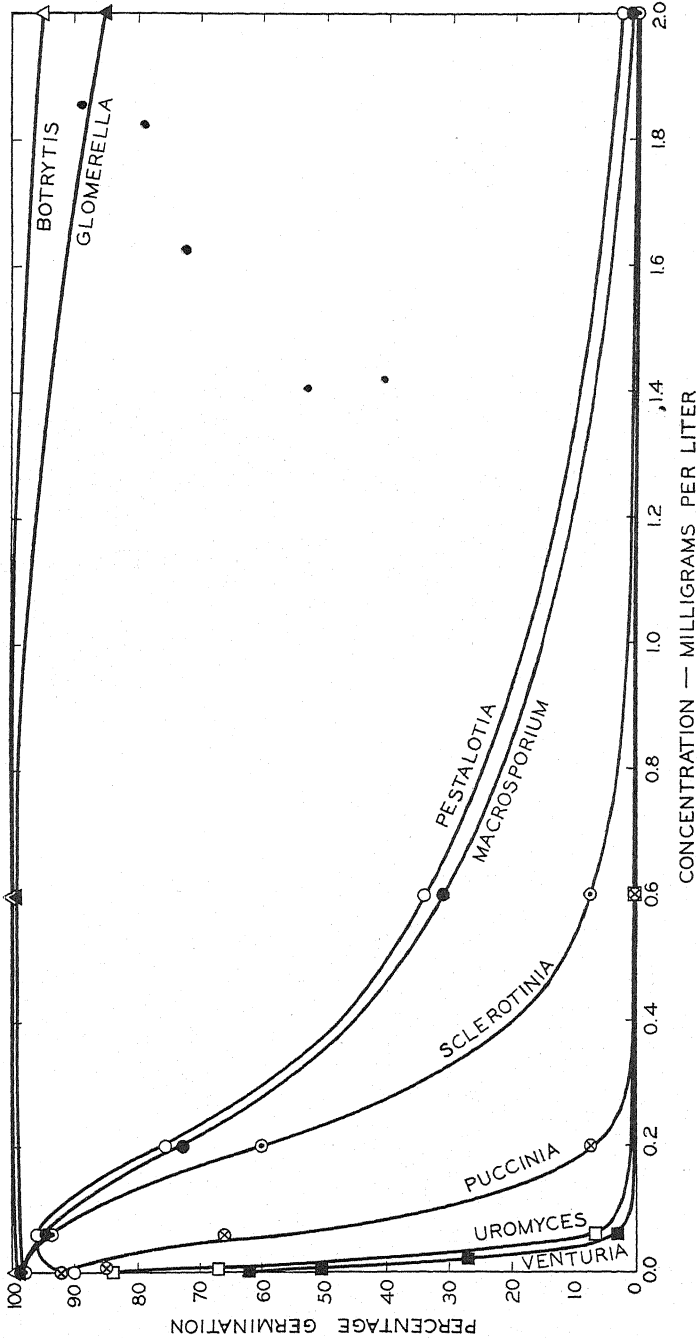


FIGURE 7. The toxicity of hydrogen sulphide to the uredospores of *Uromyces caryophyllinus* and *Puccinia anthrini*, and to the conidia of *Venturia inaequalis*, *Sclerotinia americana*, *Macrosporium sarcinaeforme*, *Pestalotia stellata*, *Glomerella cingulata*, and *Botrytis* sp. (*cinerea* type).

might be due in part to its oxidation products, namely, sulphur and sulphuric acid. In order to determine if this was the case the hydrogen ion concentration of the spore suspension drops was determined at the end of the experiment. In all cases the pH of the drops was found to be between 6.5 and 7.0, hence the amounts of sulphuric acid formed are insufficient to account for the toxicity observed, since it has been previously shown (40) that sulphuric acid does not become toxic until a pH of about 4 has been reached. When hydrogen sulphide undergoes atmospheric oxidation, finely divided sulphur appears as a product on the surface of objects exposed to the gas. Glass slides were placed in moist chambers and 20 cc. of hydrogen sulphide gas, an amount about equal to the highest concentration used in the toxicity tests, was admitted. The slides remained in this atmosphere for the usual length of time of 22 hours and were then removed to fresh moist chambers and a spore germination test performed with the slides, using conidia of *Sclerotinia americana*. There was no appreciable reduction in germination. It is evident, therefore, that the toxicity of hydrogen sulphide is due to the compound itself and not to any of its oxidation products.

#### THE CORRELATION OF HYDROGEN SULPHIDE SENSITIVITY WITH SULPHUR SENSITIVITY, AND WITH HYDROGEN SULPHIDE PRODUCTION

Since it has been shown that hydrogen sulphide is highly toxic to fungous spores and that different species vary greatly in their sensitivity to this gas, it appeared desirable to compare the sensitivity of each of the species to hydrogen sulphide and to sulphur.

TABLE VII

THE DEGREE OF SENSITIVITY TO 300-MESH DUSTING SULPHUR EXHIBITED BY THE SPORES OF EIGHT SPECIES OF FUNGI

Species	Spore form	Per cent germination		Length of germ tubes ( $\mu$ )	
		Control	Sulphur	Control	Sulphur
<i>Venturia inaequalis</i> . . . . .	conidia	66.0	20.0	125	30
<i>Uromyces caryophyllinus</i> . . . . .	uredospores	83.2	35.6	400	70
<i>Puccinia antirrhini</i> . . . . .	uredospores	90.9	57.2	400	50
<i>Sclerotinia americana</i> . . . . .	conidia	98.2	60.2	400	80
<i>Macrosporium sarcinaeforme</i> . . . . .	conidia	98.4	98.1	400	220
<i>Pestalotia stellata</i> . . . . .	conidia	89.9	89.6	300	250
<i>Glomerella cingulata</i> . . . . .	conidia	98.4	98.5	160	160
<i>Botrytis</i> sp. (cinerea type) . . . . .	conidia	99.0	99.0	150	150

These eight fungi were primarily selected for study because of their varying degree of sulphur sensitivity, which is illustrated in Table VII, compiled from a large number of experiments. From the data of Tables VI and VII the diagrams shown in Figure 8 have been prepared. In the case

of the diagrams representing hydrogen sulphide toxicity, the concentration of 0.2 milligrams per liter was selected for comparison because at this concentration the average germinations of all species approximately equaled the average germinations in sulphur dust.

Examination of this figure shows that the order of sensitivity of these eight species to sulphur and to hydrogen sulphide is exactly the same. The odds against the occurrence of such a correlation by chance are 40,320 to 1 (11).

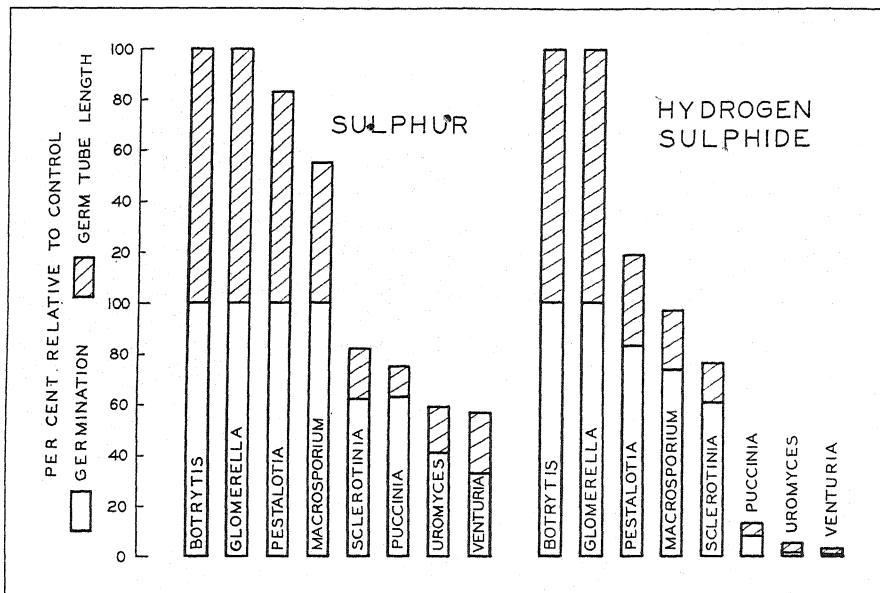


FIGURE 8. The correlation between the toxicity of sulphur dust and hydrogen sulphide solution (0.2 mg./l.) to the conidia of *Botrytis* sp. (*cinerea* type), *Glomerella cingulata*, *Pestalotia stellata*, *Macrosporium sarcinaeforme*, *Sclerotinia americana*, and *Venturia inaequalis* and to the uredospores of *Puccinia antirrhini* and *Uromyces caryophyllinus*.

It might be expected that there would be a relationship between the sensitivity of a given species to sulphur and the ability of that species to produce hydrogen sulphide in contact with sulphur, the more sensitive species producing the greater amounts. That this is not the case will be seen by reference to column 3 of Table III where the production of hydrogen sulphide is expressed on the basis of spore weight, nor does it hold true if the production is compared on the basis of spore number as is shown in column 3 of Table VIII. When, however, the production by the spores is expressed in units equal to the amount of hydrogen sulphide required to reduce germination 50 per cent a relation becomes clearly apparent, as is shown in Table VIII. The four sulphur-sensitive species which can be

controlled in the field produce more than one of these toxic units while the four resistant species which are not controllable by sulphur in the field produce considerably less than one toxic unit of hydrogen sulphide.

TABLE VIII

THE COMPARISON BETWEEN THE TOXICITY AND THE PRODUCTION OF HYDROGEN SULPHIDE, EXPRESSED IN UNITS EQUAL TO THE AMOUNT OF HYDROGEN SULPHIDE REQUIRED TO REDUCE GERMINATION 50 PER CENT

Species	Mg. H <sub>2</sub> S required to reduce germination of 1,000,000 spores 50%	Mg. H <sub>2</sub> S produced by 1,000,000 spores in 12 hours	Production of H <sub>2</sub> S expressed in units equal to the amount of H <sub>2</sub> S required to reduce germination 50%
<i>Venturia inaequalis</i> . . . . .	0.001	0.002	2.0
<i>Uromyces caryophyllinus</i> . . . . .	0.002	0.019	9.5
<i>Puccinia anthrini</i> . . . . .	0.006	0.013	2.2
<i>Sclerotinia americana</i> . . . . .	0.013	0.039	3.0
<i>Macrosporium sarcinaefome</i> . . . . .	0.043	0.013	0.30
<i>Pestalotia stellata</i> . . . . .	0.049	0.001	0.02
<i>Glomerella cingulata</i> . . . . .	0.523	0.027	0.05
<i>Botrytis</i> sp. ( <i>cinerea</i> type) . . . . .	0.665	0.002	0.003

## DISCUSSION

In presenting any theory to explain the fungicidal action of sulphur cognizance must be taken of the following experimentally determined facts. Leaves and spores of all species of plants tested produce hydrogen sulphide when in contact with sulphur. The production of hydrogen sulphide by spores varies according to the species and is directly proportional to the number of spores. The optimum temperature is 35° C. and inhibition takes place at 55° C. but the production occurs over a relatively wide pH range. The maximum rate of production at 30° C. is reached in about three hours. Actual contact between spores and sulphur is not necessary as action will take place through a collodion membrane, the hydrogen sulphide being produced on the spore side of the membrane and not on the sulphur side. The action also takes place across an air space. (Sulphur will also act at a distance on bright silver wire forming silver sulphide). The action of spores on the sulphur is primarily a reducing one. Hydrogen sulphide is extremely toxic to fungous spores, much more so than any other sulphur acid. The order of sensitivity of the spores of different species to sulphur and to hydrogen sulphide is identical and the sensitive spores produce more than one toxic unit of hydrogen sulphide, while the resistant ones produce considerably less than one toxic unit. Finally, compounds such as glutathione, which contain the -SH group, readily react with sulphur producing hydrogen sulphide at ordinary temperatures, and such compounds have been shown to exist in fungous spores.

The following interpretation of these facts as related to the fungicidal action of sulphur is offered. Sulphur in the vicinity of fungous spores, by reason of its vapor pressure, gives off sulphur vapor which diffuses into the spores. Here reduction takes place within the spores with hydrogen sulphide as a final product. The reaction is enzymatic in nature and is probably concerned with -SH compounds. The toxic product, hydrogen sulphide, being produced in intimate contact with the living cell is able to exert its maximum effect. It is not believed that the hydrogen sulphide produced from the leaves in the open affects the spores, nor that the hydrogen sulphide produced by one spore has much effect on another spore at a distance. Each individual spore, therefore, by reason of its ability to reduce sulphur to hydrogen sulphide is thus instrumental in bringing about its own death.

#### SUMMARY

1. A review of the literature is presented, dealing with the production of hydrogen sulphide from living tissues in association with sulphur, including a discussion of the widely distributed compound glutathione which is sulphur-reducing.

2. All species of plants tested were found to evolve hydrogen sulphide when in association with sulphur. The material included the attached leaves of 26 species of higher plants, the spores of 16 species of fungi, the sporophores of three species of Agaricaceae and also the expressed and filtered juice of *Pleurotus ostreatus*.

3. A quantitative study of sulphured strawberry plants shows that at 35° C. 0.002 mg. hydrogen sulphide is produced per hour per sq. dm. of leaf surface.

4. Quantitative determinations have been made of the hydrogen sulphide production by the spores of the following species: *Venturia inaequalis*, *Uromyces caryophyllinus*, *Puccinia antirrhini*, *Sclerotinia americana*, *Macrosporium sarcinaeforme*, *Pestalotia stellata*, *Glomerella cingulata*, and *Botrytis* sp. (*cinerea* type).

5. The form of the rate curves for hydrogen sulphide production has been determined and from these the total amount produced has been calculated. The total amount produced is directly proportional to the number of spores and varies with the different species. In 12 hours at 30° C., the total production varies from 9.8 per cent of the weight of the spores in the case of *Glomerella* to 0.14 per cent in the case of *Macrosporium*.

6. The effect of temperature on the rate curves has been investigated. The optimum temperature is about 35° C., and the reaction is entirely inhibited at 60° C. These temperature relations indicate an enzymatic reaction.

7. The production occurs over a pH range from about 4.0 to 8.0 with no well defined optimum.



8. It has been shown that actual contact between the sulphur and spores is not necessary for the production of hydrogen sulphide. The reaction can take place through a collodion membrane or even across an air space of 3 or 4 mm., the hydrogen sulphide being produced on or within the spores and not on the sulphur.

9. The presence of a substance containing the sulphur-reducing -SH group has been demonstrated in the spores of *Sclerotinia americana* by means of the sodium nitroprusside test.

10. Hydrogen sulphide has been found to be highly toxic to the spores of these eight fungi, the toxicity varying with the different species. When these eight species are compared as to their sensitivity to hydrogen sulphide and to sulphur the order is identical. The order of decreasing sensitivity is as follows: *Venturia*, *Uromyces*, *Puccinia*, *Sclerotinia*, *Macroporium*, *Pestalotia*, *Glomerella*, and *Botrytis*.

11. When the production of hydrogen sulphide by these spores is expressed in units equal to the amount of hydrogen sulphide required to reduce their germination 50 per cent the following relation appears. The four sulphur-sensitive species which can be controlled in the field with sulphur produce more than one unit, while the four resistant species not controllable by sulphur produce considerably less than one toxic unit of hydrogen sulphide.

12. An hypothesis is presented, consistent with the above facts, to account for the fungicidal action of sulphur. This hypothesis involves the diffusion of sulphur vapor from the sulphur particles to the spores and its reduction within the spore to form toxic hydrogen sulphide.

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MORPHOLOGICAL AND CYTOLOGICAL STUDIES ON THE  
SALIVARY GLANDS AND ALIMENTARY TRACT OF  
*CICADULA SEXNOTATA* (FALLEN), THE CARRIER  
OF ASTER YELLOWS VIRUS<sup>1,2</sup>

IRENE D. DOBROSKY

INTRODUCTION

The study of medical entomology was given a great impetus by the discovery of the rôle played by certain mosquitoes in the transmission of malaria and filaria. Within the last two decades another branch of entomology has arisen by a parallel discovery of the relationship of insects to the spread of plant diseases. The present paper reports the results of a detailed study of the morphology and cytology of certain organs of the insect which is the specific carrier of aster yellows virus. The study was undertaken in the hope that morphological or cytological differences might be found between virus-bearing and nonvirus-bearing leafhoppers. In another paper the author has discussed at greater length the disease relationships of this insect (6).

The transmission of virus diseases of plants by sucking insects does not seem to be merely a mechanical process. In several cases it has been shown that the virus must first be obtained from a diseased plant by its specific insect vector and must undergo a certain incubation period in the insect before it can infect another plant. Severin (14) showed that the minimum incubation period of the virus of curly top of sugar beet in the leafhopper, *Eutettix tenellus* (Baker), was four hours at a mean temperature of 100° F. One of the clearest examples of the necessity of an incubation period is that shown by Kunkel (11) to obtain in the case of aster yellows virus and its carrier, *Cicadula sexnotata* (Fallen). He shows that an incubation period varying from 10 to 19 days is necessary for the virus in the insect. A more recent case is that contributed by Storey (16) on the transmission of maize streak by the leafhopper, *Balclutha mbila* Naudé. His experiments showed that the minimum incubation period of the virus in the leafhopper was 6 to 12 hours at 30° C. He also found that an infected insect usually retains its infective power throughout the course of its life.

<sup>1</sup> Dissertation presented in partial fulfillment of the requirements for the degree of Doctor of Philosophy in the Graduate School of Cornell University, September 1928. A joint contribution from the Graduate School of Cornell University, Ithaca, New York, and the Boyce Thompson Institute for Plant Research, Inc., Yonkers, New York.

<sup>2</sup> The writer wishes to acknowledge her indebtedness to Dr. O. A. Johannsen of Cornell University under whom she received her training in morphology, and to members of the staff of the Boyce Thompson Institute for unfailing interest from the inception of the work to the reading of the manuscript.

In medical entomology, the biological transmission of diseases by insects has been carefully studied for a number of diseases. The yellow fever virus must undergo a 12-day incubation period in its insect host, *Aedes aegypti* L. The protozoan, *Plasmodium falciparum* Welch, associated with tertian malaria, will complete its development in its anopheline host in one to three weeks depending on the temperature. In the case of Nagana disease, the causative organism, *Trypanosoma brucei* Plimmer & Bradford, requires ten days or longer for its incubation period in the tsetse fly, *Glossina morsitans* Westwood. The sleeping sickness organism, *Trypanosoma gambiense* Dutton, requires several weeks' development in its essential host, *Glossina palpalis* Robineau-Desvoidy.

All these diseases, except perhaps yellow fever, are definitely linked up with some visible protozoan parasite. In the virus diseases of plants and animals, however, the etiological agent is not known. There are two widely differing opinions as to the nature of the virus. One group of scientists considers the virus to be of a purely chemical nature. The other group believes the virus to be an organized living entity, which is either of extremely small size or else is so plastic that it can pass through the pores of a fine filter. Looking at it from the entomological point of view, it seems most probable that the virus is a living organism. Storey (16), as a result of his experiments, expresses this opinion as follows: "Thus, it can hardly be questioned that some specific biological relationship exists between *Balclutha mbila* and the streak virus. The evidence clearly indicates a multiplication of the virus in the insect. While it may be argued that the insect in reality is merely becoming repeatedly reinfected by virus which has multiplied in the plant at the point of feeding, yet this is not a reasonable criticism, in view of the evidence presented of the permanence of the infective power of the leafhopper under different conditions. The incubation period in the insect admits of two explanations; either it is the time taken for the virus to move from the gut to the salivary glands and perhaps also to multiply up to the point where an infective dose passes out with the saliva; or possibly the virus undergoes a cycle of development within the insect."

A review of literature on insect-borne diseases reveals the fact that the salivary glands are the usual locus of the parasite in its host. The first case discovered was that of the malaria organism. Cysts filled with the organism are formed in the stomach cells of the mosquito and when these cysts break the organisms penetrate into the salivary glands. There are some excellent colored plates by Wenyon (18) showing these stages. A *Herpetomonas* associated with oriental sore was found in the salivary glands of the fly, *Phlebotomus papatacii* Scop. (1, 2). *Trypanosoma brucei* and *T. gambiense* are said to invade the salivary glands of their respective insect hosts by the migration of the intestinal form to the proboscis and

thence up to the salivary glands. Organisms associated with plants have also been discovered in the salivary glands of insects which feed on these plants.

França (9) found the flagellate, *Leptomonas davidii* Lafont, in the glands of the hemipteron, *Stenocephalis agilis* (Scop.), which feeds on the *Euphorbia* which harbors this flagellate. Holmes (10) found *Herpetomonas elmassiani* (Migone) in the salivary glands of the bug, *Oncopeltis fasciatus* (Dall.), which transmits this flagellate to milkweeds. Moreover, he found that the flagellate was localized in a certain portion of the salivary glands. When staining the glands with iron haematoxylin and eosin, he found that a certain lobe which took the deepest red stain was the one which contained the flagellates. This points to a high degree of specificity on the part of the parasite.

Little comparative work has been done on the histology and cytology of normal and viruliferous insects. The leafhopper, *Cicadula sexnotata*, was chosen for such a study, because in this insect the virus of aster yellows must undergo a period of development of at least ten days before it can infect a plant. Incidentally, this species is very abundant during the summer, and can also be reared continuously during the winter in a greenhouse. As a review of medical and phytopathological literature shows, obligate parasites are usually found in the salivary glands of alimentary tract of their hosts. An intensive study of the salivary glands and alimentary canal of *Cicadula sexnotata* was, therefore, undertaken.

#### METHODS

Paraffin sections of more than a thousand healthy and virus-carrying insects, reared in a greenhouse, were made. The fixatives used were Gilson's, Bouin's, Regaud's, Carnoy's, and Flemming's Weak. The sections were cut to a thickness of 4, 5, or 7.5 $\mu$ . They were then stained with Wright's; Heidenhain's haematoxylin and eosin; Flemming's triple; haematoxylin and Congo red; Giemsa's; Pianese III b; Mallory's; gentian violet and safranin; or acid fuchsin. The best results for salivary glands were obtained with Gilson's fixative followed by Wright's or Wolbach's modified Giemsa stain. Bouin's fixative followed by haematoxylin and eosin was found excellent for nuclei, but not for cytoplasm. By using slides made with the Gilson-Wright combination for texture of the cytoplasm and the Bouin-haematoxylin combination for nuclei, satisfactory material for the study of the salivary glands was obtained.

#### SALIVARY GLANDS

The salivary glands are made up of three pairs of glands, the principal portion consisting of two pairs closely united at a common point where they are joined by the duct of the small accessory glands. A comparative



FIGURE 1. A. Longitudinal section of adult female leafhopper, showing wing muscle in thorax, and filter-chamber, crop, ascending mid-intestine, one egg follicle, and malpighian tubules in abdomen. (Gilson's fixative, Wright's stain.  $7\mu$  thick.  $\times 50$ .) B. Longitudinal section of adult female leafhopper, showing salivary glands in prothorax, and mycetome on either side of the first and second abdominal segments, and two egg follicles in upper center of abdomen. (Gilson's fixative, Wright's stain.  $7\mu$  thick.  $\times 50$ .)

study of related forms shows that this is the primitive condition. Dissections of the 17-year cicada plainly show these three pairs of glands. In aphids there are usually two pairs and occasionally three. According to Bugnion and Popoff (4), a study of aphids proves that in the primitive condition the glands were formed of three lobes, of which two are contiguous, and the third more or less detached and elongated. In the closely related family Fulgoridae, there is a species of *Fulgora* which also has three pairs of glands corresponding to the anterior and posterior lobes of the principal gland and the accessory gland of the Heteroptera. This accessory gland is a long sinuous organ used for storage purposes in the Heteroptera. That it should be present, though poorly developed in some Homoptera, shows the close relationship between these two orders of insects.

#### MORPHOLOGY

The salivary glands in *Cicadula sexnotata* lie beneath the brain and occupy the dorsal floor of the head. They fill almost the whole of the prothorax and extend for a short distance into the mesothorax (Fig. 1, B).

They consist of two equal halves which lie on either side of the oesophagus. A wax model made to represent the principal glands resembled in appearance the kernel of a walnut. The average measurements of the salivary glands are  $360\mu$  in length,  $380\mu$  in width, and  $270\mu$  in depth from dorsum to ventrum. Figure 2, A-F and Figure 3, A-F show serial sections of the salivary glands from which the wax model and measurements were made.

Each pair of the principal glands is made up of about 21 lobules or acini which are closely appressed, sessile, and joined to a common central duct by short individual ducts. In arrangement, the acini of the lower portion (Fig. 4, I-IV) which are mucous glands, resemble a partially opened rose. The shape of each acinus depends on its position, those in the center being the smallest, the middle ones long and foliaceous, and the outermost large and thick. The upper acini (Fig. 4, V) which are serous glands, are arranged like a pinnately compound leaf. There are seven acini, the topmost being single and the others aligned on either side of a central duct.

The accessory gland consists of four cuboidal cells on the tip of a short thick duct which joins the common duct of the principal glands at its point of departure from the acini. The length of the entire gland, from the tip of the secreting cell to where it merges with the Type V acini, is not more than  $117\mu$ . The glandular tip is  $11\mu$  in diameter. The small size of these glands seems to indicate their vestigial nature.

An excretory duct leads from each half of the glands toward the beak where the two unite to form a common duct. The latter opens on the floor of the hypopharynx into the salivary pump. The inner diameter of each



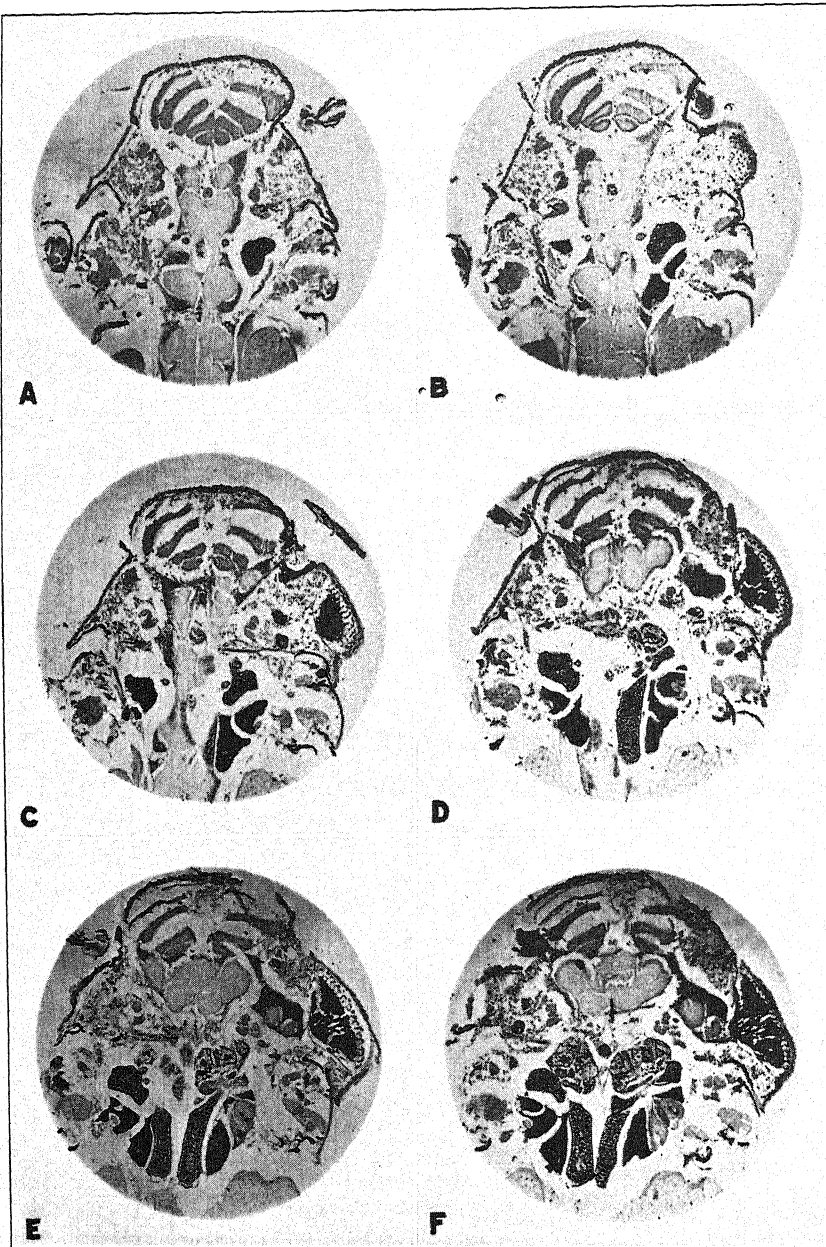


FIGURE 2. A-F. Serial sections of the salivary glands of *Cicadula sexnotata*. Note the two round cross sections of the salivary ducts on either side of the nervous system in Figures A-C. (Gilson's fixative, Wright's stain.  $7\mu$  thick.  $\times 90$ ).

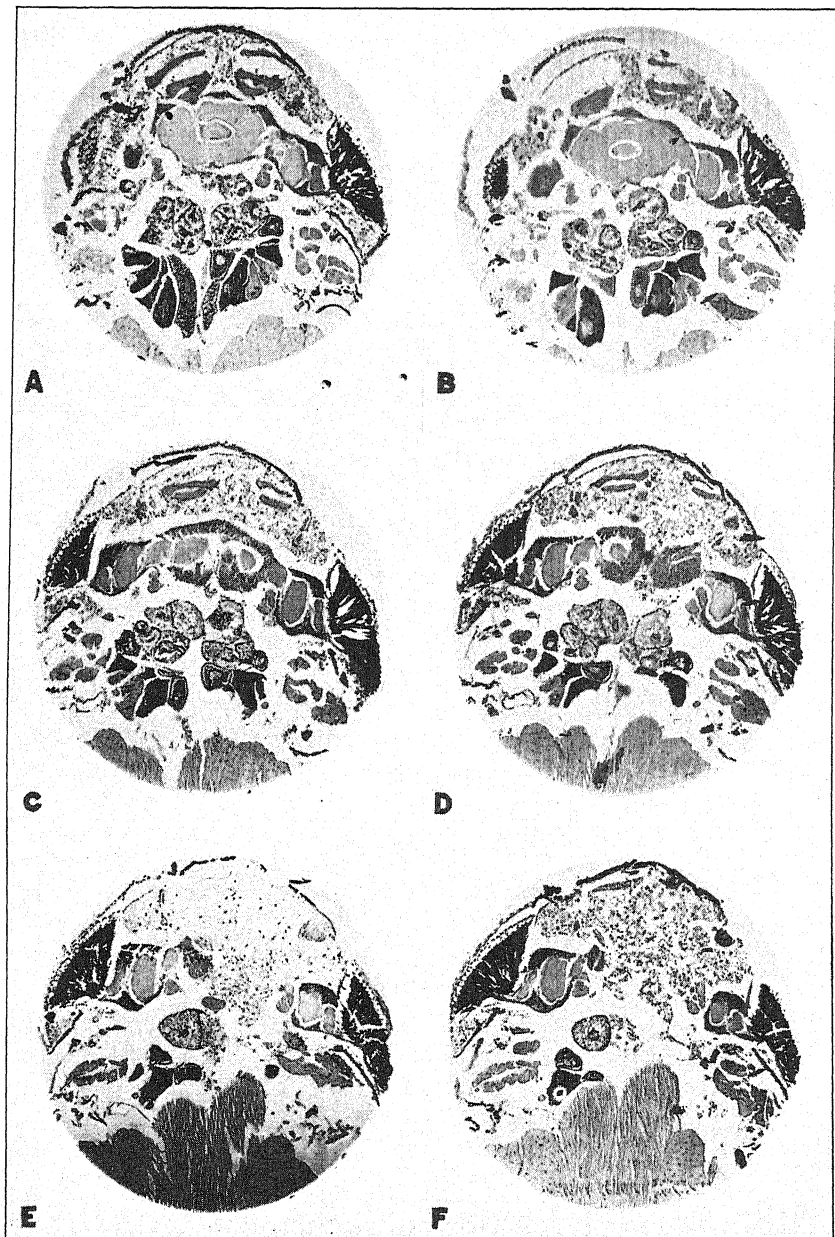


FIGURE 3. A-F. Continuation of serial sections of salivary glands of *Cicadula sexnotata*. (A is illustrated in Fig. 6). (Gilson's fixative, Wright's stain.  $7\mu$  thick.  $\times 90$ ).

duct as it leaves the salivary gland is  $2\mu$ , the outer  $4.5\mu$ . The common duct at its point of formation in the hypopharynx is  $2.7\mu$  at its innermost and  $7.2\mu$  at its outermost diameter.

As the common duct of the salivary glands approaches the pharynx, it enters a small barrel-shaped organ, termed the salivary pump (Fig. 6, C). This organ is about  $27\mu$  at its widest and  $39\mu$  at its longest dimension. It consists of a highly chitinized cylinder and piston. The latter is a pear-shaped inverted portion which almost fills the cylinder, the inner chamber of which can be seen as two horn-shaped spaces on either side of the piston. Above the salivary pump the salivary duct can be seen at the point of en-

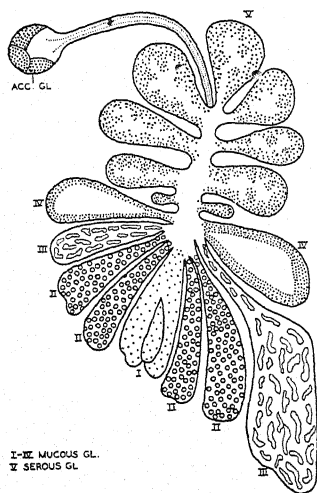


FIGURE 4. A schematized drawing of the salivary glands of *Cicadula sexnotata*.

trance to the pump. Tower (17) in his paper on the mouthparts of the squash bug explains in detail the manner in which such a pump as this works.

Before proceeding with the cytology of the gland cells, a brief review of the subject of secretion is desirable.

There are at least three distinct variations in the process of cell secretion. In the one type the whole cell is destroyed and is replaced by an entirely new cell. This is true of the sebaceous glands. In another type a portion of each cell is used up in the production of the secretion, only the basal portion containing the nucleus being preserved. When this occurs, as it does in the mammary glands, the upper part of the cell is reconstructed from the remaining basal portion, and the cell is ready to renew the process. In the third type the cell remains intact throughout the process, as illustrated by the salivary glands.

The two physiological types of salivary gland cells are readily distinguishable from one another. The mucous variety secretes a viscid fluid containing mucin, and the serous variety secretes a thinner and more watery fluid. The microscopic appearance of the gland cell varies according to the functional condition of the cell. In the fresh state the mucous cells contain numerous large granules of mucinogen. This is especially striking if the insect has been starved before fixation; in this case, the serous glands also are large and distended with a thin faintly-staining secretion.

#### CYTOLOGY

The acini of the salivary glands seem to be single cells in spite of the fact that they are binucleate. Cholodkovsky (5) in his study of *Chermes* brings forth the theory that at one time each acinus was two-celled. He bases this on the fact that the accessory glands consist of two cells which are uninucleate. This was not borne out in the study of the accessory glands of the leafhopper in question.

The appearance and stainability of the salivary glands differed with the different fixatives. Bouin's fixative followed by Heidenhain's haematoxylin and eosin was found excellent for nuclei, but not for cytoplasm. Carnoy's and Flemming's Weak caused the tissues to swell and tear. Salivary glands fixed in Flemming's Weak and stained with the Triple stain are shown in Figure 6, F. Regaud's gave good results and took the aniline dyes very well. Bouin's did not permit the aniline dyes to stain the glands well. Gilson's gave the best results and combined well with all the dyes. Glands fixed in Gilson's and dyed with iron haematoxylin and eosin are shown in Figure 6, E.

*The mucous glands.* The mucous glands are those labelled I, II, III, and IV in Figure 5. This drawing is a careful reproduction of the cytological aspect of the different acini. The glands to be described were fixed in Gilson's and stained with Wright's blood stain. With this aniline dye the mucin is colored red. Tests with safranin and with iron haematoxylin corroborate the fact that the lower acini contain mucin, whereas the upper acini do not.

The mucous glands consist of four very distinct types of cells. Type I cells are the smallest and are in the center of the rose-like conformation of acini. There are about five cells of this type. Their cytoplasm is finely granular and homogeneous in texture. It takes a faint grayish-blue stain. The two nuclei are small, compact, and stain a deep blue. These details are well illustrated in Figure 6, E.

Surrounding these central cells are five acini of Type II. In size, texture, and arrangement they differ markedly from the preceding type. They are larger and filled with round red globules of mucinogen. The cytoplasm around the globules stained blue. Two large nuclei occupy the center of

each acinus. They are larger than the nuclei in Type I acini, their contents are not as compact, and stained a deep blue.

There are only two cells of Type III. They are by far the largest of the acini. One of these two acini is the largest cell in the insect's body, with the exception of the mature ovum. This large acinus measures  $240\mu$  in length and  $60\mu$  in width. It is contiguous to the oesophagus. The other cell of this type is found on the further side of the Type II acini. Their

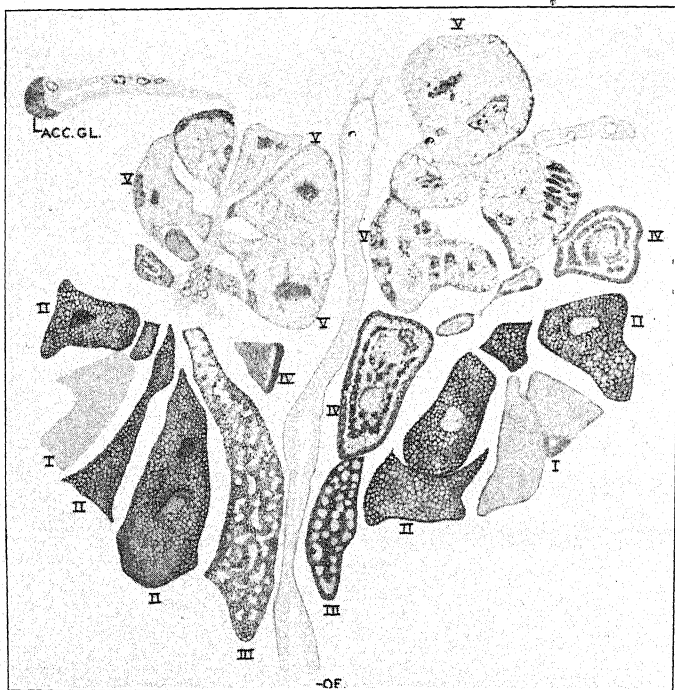


FIGURE 5. Drawing of salivary glands of adult leafhopper showing the five types of acini. (Camera lucida drawing of Fig. 3, A). ACC. GL.—Accessory gland; OE.—Oesophagus; I—Type I acini; II—Type II acini; III—Type III acini; IV—Type IV acini; V—Type V acini.

cytoplasm stains a light blue and is filled with long sinuous vacuoles containing a homogeneous pink-staining secretion. The two nuclei, centrally placed one above the other, are extremely large. Their chromatin stains a light greenish-blue and in appearance is like short bacterial rods, scattered evenly throughout. There is no nucleolus present.

Type IV acini are two in number and lie on the outside of each of the Type III acini. Their cytoplasm is scattered and vacuolate in appearance. Usually it is disposed in a dense blue-staining border around the periphery.

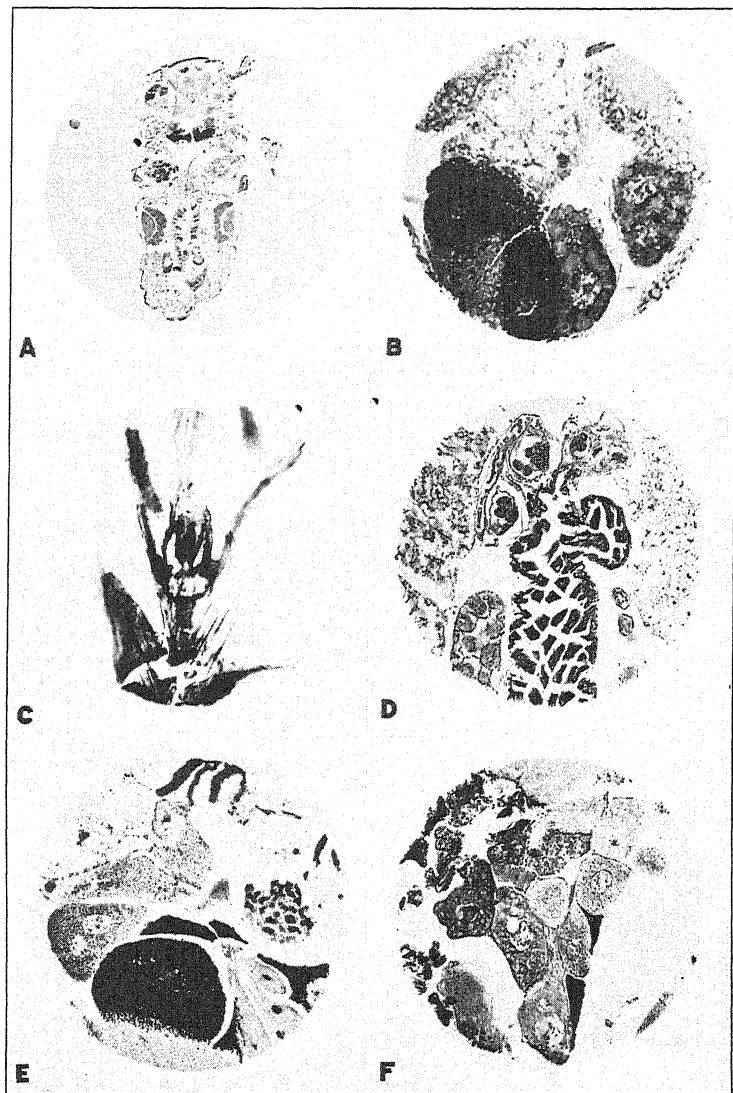


FIGURE 6. A. Longitudinal section of five-day-old nymph of *Cicadula sexnotata*, showing salivary glands, alimentary tract, and mycetome. (Gilson's fixative, Wright's stain.  $7\mu$  thick.  $\times 80$ .) B. Salivary glands of one-day-old nymph, showing acini of Types I, II, III, and V. Note large size of mucinogen granules in Type II. (Gilson's fixative, Wright's stain.  $7\mu$  thick.  $\times 550$ .) C. Salivary pump of *Cicadula sexnotata*. D. Portion of alimentary tract showing filter-chamber, crop, and part of ascending mid-intestine. (Gilson's fixative, Wright's stain.  $7\mu$  thick.  $\times 180$ .) E. Salivary glands of *Cicadula sexnotata* showing five types of acini. (Gilson's fixative. Iron haematoxylin eosin stain.  $7\mu$  thick.  $\times 270$ .) F. Salivary glands of *Cicadula sexnotata* fixed in Flemming's Weak and stained with safranin and gentian violet.  $7\mu$  thick.  $\times 190$ .)

In some cases there is a clear zone between this peripheral layer and the inner cytoplasm. The two nuclei are large and in structure and staining properties resemble those of Type III acini.

*The serous glands.* The upper half of the salivary glands consists of seven more or less similar cells which form the serous glands. These acini are designated as Type V. The upper acini are large and very vacuolate. They contain but little cytoplasm which is found scattered irregularly throughout the cell and around its periphery. The two nuclei are small, of irregular outline, compact, and darkly-staining. The two lowermost cells are much smaller and their homogeneous cytoplasm takes a faint pink coloration. The nuclei are large, regular in shape, and not as compact as those of the other acini of this type. The seven acini all join into a narrow pink-staining central area which contains many large, faintly-staining nuclei.

From the appearance and arrangement of the acini and these numerous nuclei, one might postulate a cycle of origin, growth, and deterioration for these acini of Type V. The presence of these nuclei at the base, the small size and homogeneous appearance of the two lowermost acini, and the vacuolate, scattered appearance of the topmost acini all point to the probability that these acini arise at the base, increase in size, and then break down when they attain their full size and have functioned in excretion.

*The accessory glands.* The accessory glands consist of four cuboidal cells at the end of a short thick duct. These cells take a very deep blue stain. They contain large vacuolate nuclei which almost fill them. The accessory duct is made up of faintly pink-staining cells, closely adjoining and flattened out. Each of the cells contains two faintly blue-staining nuclei. The duct is seen to enter between the central cell of Type V and its neighbor and meets the duct of the principal gland at the common center of all the acini (Fig. 5).

*The salivary ducts.* The ducts which issue from each half of the salivary glands to unite in the hypopharynx resemble large tracheae. They are lined, as are the tracheae, with spirally thickened intima. The walls are made of flattened cells which contain very large nuclei. A cross section of the duct shows the lumen surrounded by a thick intima which in turn is encased in a thick pink-staining layer of epithelium. Fine striations radiating from the center are clearly visible in the cytoplasm. Occasionally a large blue-stained round nucleus is encountered.

#### SALIVARY GLANDS OF THE NYMPH

A comparative study of the salivary glands of the adult and nymph was made in order to determine certain points, as to the origin, growth, and fate of the acini. The salivary glands of nymphs of all instars and especially those of nymphs from one to five days old were studied. In a one-day-old

nymph (Fig. 6, A) which was 0.87 mm. long, the principal glands measured  $136\mu$  in length and  $130\mu$  in width. They were found to consist of the same number of acini as in the adult and were in the same arrangement. The accessory glands were present and corresponded to those found in the adult.

Acini of Type I are small and crinkled in appearance. The cytoplasm stains a faint blue and contains small compact nuclei.

Type II acini, five in number, are very striking in appearance. They are so tightly packed with large deep red globules, that they bulge. These globules of mucinogen are noticeably larger than those in adults. Two compact dark blue nuclei are present in each acinus.

The two acini of Type III are the largest of the acini. Their cytoplasm takes a faint blue stain and contains a few very large convoluted vacuoles which are filled with pink-staining mucin. Two large nuclei are present which are a faint grey in color, and contain chromatin scattered in loose granules.

Type IV acini, two in number, are almost round in shape. The cytoplasm is coarsely reticulate and forms a faint narrow clear zone close to the periphery. There are two very large granular nuclei with scattered rod-like chromatin.

The Type V acini, or the serous glands, are seven in number. Their cytoplasm is very vacuolate, and distributed mostly around the periphery. The nuclei are of medium size and rather compact.

The accessory gland is proportional in size to the principal gland. It consists of four cuboidal cells at the tip of a short thick duct, lined with intima and with a layer of large cells in which are embedded several large nuclei.

These cytological details are shown in Figure 6, B. Note the globules of mucinogen in Type II acini, the large lakes of mucin in Type III acini, and the scattered, vacuolate appearance of Type V acini.

Comparing the acini of the nymph with those of the adult the following differences may be noted:

1. Type II cells are more distended with larger granules of mucinogen.
2. Type III cells have larger and fewer vacuoles.
3. Type V cells have less cytoplasm and are more vacuolate than in the adult.
4. All the nuclei are much larger proportionally in the nymph than in the adult.

The following likenesses may also be noted:

1. The number of acini is the same.
2. The arrangement of acini is the same.
3. The size of the salivary glands is in proportion to the size of the insect.

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4. The function of each acinus as shown by its appearance seems to be the same.

5. The nuclei in each corresponding acinus seem to be in the same state of activity.

To find the origin of each acinus it would be necessary to study the embryological stages of the insect. The salivary glands in the youngest nymph were found to correspond rather closely to those of the adult.

Berlese (3), studying the glands of aphids, says that there are marked variations of the cellular content and especially of the nucleus in the process of secretion. He then says that the cytoplasm is first of a homogeneous aspect and later becomes very reticulate and has droplets in its network. A carefully study of the glands of over a thousand leafhoppers does not show the process as described by Berlese. Each acinus, even in a one-day-old nymph, always presents the same general aspect, and can be determined as being Type I, II, III, IV, or V.

Dufour (7, 8) regarded the lower pair of salivary glands of Hemiptera as the reservoir of the upper. Staining reactions in *Cicadula* do not support this view. The basic aniline dyes used showed the presence of mucin in the lower acini but never in the upper.

#### EFFECT OF STARVATION ON SALIVARY GLANDS

In order to become acquainted with all appearances of the salivary glands, adults were starved from 10 to 26 hours. As sucking insects are almost constant feeders, it was thought that this length of time would be sufficient to bring out the effects of starvation. If the insects were kept in closed test tubes for a longer period they died either from starvation or dessication.

A comparative study with insects which were taken and fixed while in the act of feeding shows that in starved insects the salivary glands as a whole become larger, each acinus becoming more turgid and distended. The cytoplasm is finely reticulate throughout. The nuclei are much larger, round, and very deeply stained.

Cytologically the four types of acini of the mucous glands of starved individuals do not differ markedly from those of normal individuals. Type I cells are the same as normal cells. Type II cells are swollen and compactly filled with globules of mucinogen. Type III cells are long and filled with long narrow vacuoles containing mucin. Type IV cells are noticeably larger than normal. The Type V, or serous glands, are markedly different. They are very much swollen and filled with a dense homogeneous cytoplasm in the center of which are two large, round, densely-staining nuclei. Normally these acini are pear-shaped and have but little cytoplasm which is scattered around the periphery. Their two small nuclei are usually irregular in shape.

## SALIVARY GLANDS OF DISEASE-CARRYING INSECTS

An intensive study of the normal glands was necessary before it was feasible to examine the glands of disease-carrying individuals. It is possible to obtain disease-free insects by hatching them on rye. This plant is not susceptible to the aster yellows virus. Each acinus of the glands of a normal leafhopper was carefully compared with the corresponding one in an insect which had been reared on diseased aster plants. In the endeavor to find some differences, the following characteristics were studied:

1. Size, shape, and staining properties of each acinus.
2. Texture and staining reaction of cytoplasm.
3. Vacuoles and inclusions in cytoplasm.
4. Size, staining properties, and physiological state of nucleus.
5. Amount of secretion.

No constant observable difference, morphological or cytological, was found between the glands of healthy and disease-bearing individuals. Special stains for the detection of foreign organisms, such as bacteria, fungi, protozoa, rickettsia, and intracellular bodies, were used. Nothing resembling these organisms was found in the salivary glands of either healthy or disease-carrying insects.

## ALIMENTARY CANAL

It has been found in the case of several insect-borne diseases that the parasites are first localized in some part of the alimentary canal and from there migrate to the salivary glands. With this in mind, a thorough study of the entire alimentary canal was made. Figure 7 is a schematized drawing of the alimentary system.

## MORPHOLOGY

The alimentary tract of Homoptera is unique among the insect orders. As early as 1833 Dufour (7) made an extensive study of the alimentary system of *Cicada orni*. He found that the mid-intestine bends back on itself and forms a closed "boucle" or ring. The region where the ascending mid-intestine comes in contact with the fore part of the mid-intestine has been termed the "filter-chamber" by Berlese, and the "internal gland" by other authors.

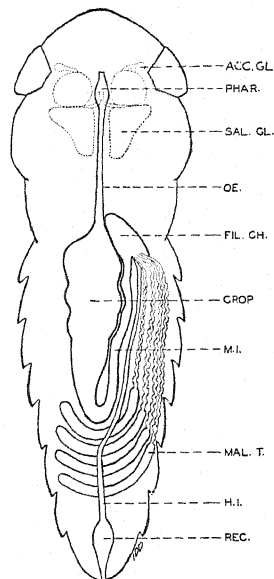


FIGURE 7. A schematized drawing of the alimentary tract of *Cicadula sexnotata*. ACC. GL.—Accessory gland; PHAR.—Pharynx; SAL. GL.—Salivary glands; OE.—Oesophagus; FIL. CH.—Filter-chamber; CROP—Crop or stomach; M. I.—(Ascending) Mid-intestine; MAL. T.—Malpighian tubules; H. I.—Hind-intestine; REC.—Rectum.

The function of this region has been under discussion for many years. This organ will be described in detail in another place.

*Mouth parts.* The mouth parts of sucking insects have been studied in great detail by many workers. The subject has received recent attention for the purpose of finding an explanation of the fact that one species of insect can transmit a virus disease and another closely related species cannot. Smith (15) studied the mouth parts and punctures of several species of Homoptera and found that some species always feed in the phloem of plants, others in the parenchyma, and still others in either region. No correlation was found between the length of the stylets and the ability to transmit the virus. He concludes that there is no simple mechanical explanation for the transmission of viruses by insects.

*Pharynx.* In *Cicadula sexnotata* a slight enlargement in the head, starting at the mouth opening and turning downward into the oesophagus, constitutes the pharynx. It is a strong pumping organ used to create suction in order to draw up the plant juices. In the fore part of the floor of this organ is found the salivary pump which opens on the hypopharynx.

*Oesophagus.* The oesophagus is a thin straight tube, starting in the prothorax and lying between the paired salivary glands. It continues with a slight increase in diameter to the first abdominal segment. This organ is purely passive, functioning only as a passage for food.

Between the oesophagus and the abrupt dilatation of the mid-intestine, known as the crop, a valve is formed by several large cells which hang downward into the crop. A slight invagination of the oesophagus also occurs at this point. This valve insures against the regurgitation of the liquid food.

*Mid-intestine.* The fore-intestine as represented by the oesophagus adjoins the mid-intestine which at that point is called the crop or stomach. The crop is a pear-shaped organ which tapers down gradually from the first to the fourth abdominal segment. At this end of the crop the mid-intestine makes a sharp U-bend upward and ascends as a narrow tube closely applied to the side of the crop. This ascending mid-intestine terminates in the first abdominal segment, forming, in part, the organ known as the filter-chamber.

According to Berlese's theory the liquid part of the food diffuses by osmosis from the stomach into the mid-intestine and then into the hind-intestine, leaving the more nutrient matters to pass into the ascending mid-intestine and more slowly find their way by the usual route to the rectum. Licent (12, 13), enlarging upon Berlese's theory, shows that the valve between the mid- and hind-intestine is the cause of the retention of the excreta. As a result, the cells of the ascending mid-intestine become tightly packed with calcium carbonate, rendering passage difficult and probably even putting the mid-intestine out of the circulation except by osmosis. Licent fed insects with colored juices and showed that the crop was the seat

of digestion and absorption. He concludes that the filter-chamber is especially adapted for the passage of liquid food by osmosis; that the ascending mid-intestine takes on the excretory function of the malpighian tubules and these in turn become transformed partially into glandular organs.

The name "filter-chamber" is good from the point of view of its function. Other authors, however, have called it the "anterior gland" and the "internal gland." These names are equally applicable judging from the morphology and cytology of the organ. As shown in Figure 6, D, there are large gland-like cells within it. The whole assembly is covered by an almost transparent peritoneal membrane which is intricately convoluted throughout the organ and contains occasional large nuclei. This chamber or gland is so closely appressed to the anterior portion of the crop that it is very difficult to distinguish between the cells of each.

*Malpighian tubules.* The four malpighian tubules arise at the junction of the mid- and hind-intestine. Two of the tubes arise singly, the other two are united for a short distance from their origin. They start as slender, undulating ducts which extend to the fifth abdominal segment where they connect abruptly with thick blind tubes which curl upward close to the U-bend of the mid-intestine (Fig. 1, A).

*Hind-intestine.* The hind-intestine is a very slender tube which resembles in structure and staining reaction the connectives of the malpighian tubules. It runs parallel with these connectives until it widens out gradually to meet the rectum in the last two segments of the abdomen. It differs from the connectives in that its walls are smooth and parallel and its cells are flat and interwoven and have an internal striated border.

The rectum is a pear-shaped organ found in the last two abdominal segments and terminates in the anus. It has very thin walls which in occasional specimens are distended with a green homogeneous substance, probably excrement. A strong layer of circular muscle envelopes the rectum, and a few longitudinal muscles are found outside the circular ones.

#### CYTOLOGY

It is a striking fact that all organs of the alimentary tract are composed of binucleate cells. This is true of its appendages, the salivary glands and the malpighian tubules, as well as of the tract proper. Both nuclei of each cell are of the same size and in the same state of activity. They seem to be of equal value to the cell. As the insect is a continuous feeder, it may be that two nuclei are necessary to aid in the process of secretion and digestion. The organs described below were fixed in Gilson's and stained with Wright's blood stain.

*Oesophagus.* Since the oesophagus serves merely as a conductor of food, its cells are extremely flattened and elongate. At rest, the oesophagus is thrown into longitudinal folds. The hypodermal cells are interwoven into

each other to make a flat inner surface which is covered with intima. Nuclei are medium sized and take a deep blue stain. The cells of the oesophageal valve are elongate and filled with a dense protoplasm. Their nuclei are large and active.

*Crop or stomach.* The crop is the organ of prime importance as the seat of digestion. Its cells are very large, rather cuboidal in shape, and contain large nuclei. A basement membrane, with small bead-like thickenings, is present. With Wright's stain a beautiful picture is obtained showing the method of secretion. The homogeneous cytoplasm takes a strong pink, the two large nuclei take a deep blue stain, and the inner border of the cell takes a definite clear purple. All the cells of the crop have this coloration but at the bend of the mid-intestine the character of the cells changes abruptly. The cells of the ascending mid-intestine take a very faint stain. Their cytoplasm is sparse and ragged and stains a faint pink with Wright's. The two nuclei are very large and have a scattered chromatin. There is no deep purple border on the inner margins of these cells. It is in this region that calcium carbonate is deposited in the cells. In the process of fixation the crystals have been dissolved. This region is clearly shown in Figure 1, A and Figure 6, D. The crop with its deeply-staining cells occupies the center of the upper part of the abdomen. Parallel and close to it is the ascending mid-intestine with its faintly-stained cells.

*Hind-intestine.* The lumen of this region is very small. The cells of the hind-intestine are cuboidal but somewhat flattened and have a deep striated inner border which stains a bright pink. Judging from the striated border the cells of the hind-intestine have a secretory function as well as the usual one. The nuclei are large and their scattered chromatin takes a bright blue stain.

*Malpighian tubules.* The long narrow connectives of the tubules are made of large cells which alternate with one another on each side as seen in longitudinal section. These cells stain a deep pink and contain large spherical nuclei. The cells of the enlarged portion of the tubules are very large and wedge-shaped. They are so closely appressed that the lumen is almost negligible. These cells are glandular in nature and stain a bright blue. The cytoplasm makes a regular network pattern denser at the base and almost transparent at the inner border. The nuclei are all basal and rather small for the size of the cell. Their chromatin is bunched into a few large masses.

*Rectum.* As the hind-intestine joins the rectum the character of the cells changes radically. The cells of the rectum are irregular in shape and are scattered at intervals over the surface of the organ. The nuclei are small, compact, and stain dark blue.

#### ABSENCE OF ORGANISMS

In a careful morphological and cytological study of the organs and cells of the alimentary canal of *Cicadula sexnotata* no parasitic organism was

found and no structural peculiarities were observed in the anatomy of the alimentary tract of virus-bearing insects. No cytological explanation of the ability of this insect to transmit the virus of aster yellows was obtained. Bacteria were observed in the lumen of the alimentary tract of only a few of more than a thousand individuals studied. This is somewhat surprising since bacteria are doubtless taken up occasionally when the insect feeds. It may be that the juices in the alimentary tract are unsuitable for bacterial growth.

#### SUMMARY

1. A comparative morphological and cytological study of virus-free and virus-bearing individuals of the leafhopper, *Cicadula sexnotata*, did not reveal the presence of any organism that could be associated with the transmission of the virus of aster yellows by this insect.

2. A morphological study of the salivary glands of *Cicadula sexnotata* shows that they are composed of three pairs of glands; the mucous, the serous, and the accessory glands.

3. A cytological study of the salivary glands shows that there are five distinct types of acini in the principal glands.

4. The salivary glands of the youngest nymphs correspond very closely in number and arrangement of acini with those of adults.

5. In insects that were starved the salivary glands were larger than normal.

6. A comparative study of the salivary glands of normal and disease-carrying individuals showed no constant observable difference.

7. No foreign organisms were found in the salivary glands.

8. All the cells of the alimentary tract and its appendages were found to be binucleate.

9. A morphological study of the alimentary tract was made to show the relationship of the different regions.

10. A cytological study of the alimentary canal shows that the crop and filter-chamber are the important points of digestion; that the ascending mid-intestine is filled with crystalline products; and that the cells of the malpighian tubules and hind-intestine are secretory in nature.

11. No foreign organisms of any description were found in the cells of the alimentary tract or its appendages. In the lumen of the alimentary tract bacteria were not observed in the vast majority of more than a thousand insects examined.

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# STUDIES ON CRANBERRY FALSE BLOSSOM DISEASE AND ITS INSECT VECTOR

IRENE D. DOBROSKY

## INTRODUCTION

The cultivated American cranberry, *Vaccinium macrocarpon* Ait., is seriously affected by a virus disease called false blossom. As the disease was first found in Wisconsin, it was called Wisconsin false blossom to distinguish it from the fungus disease caused by *Exobasidium oxycocci* Rost. which was called Massachusetts false blossom. Shear (23) proposed the name rose-bloom for the latter.

The wild cranberry is indigenous to North America and is found growing in open bogs and swamps from Newfoundland, westward to Wisconsin and southward to West Virginia and Arkansas. The plant is cultivated extensively in Nova Scotia, Massachusetts, New Jersey, Long Island, Wisconsin, Oregon, and Washington. Franklin (10) estimated that there are 30,000 acres devoted to this crop; but if the land used for roads, sand banks, and reservoirs were included, it would amount to 70,000 acres. In 1907, Shear (20) estimated the crop to be worth \$2,000,000 annually. For 1929, Stevens estimates it roughly at \$6,000,000.

Cranberry bogs have been known to bear crops for 75 years without being replanted. Fungus diseases (24) may ruin the crop for a year or two, but the false blossom disease renders the plant nonproductive for the remainder of its life. The plant does not die as a result of the disease but remains a source of infection and danger for the healthy plants. Moreover, a plant once diseased remains so and does not recover (27). The spread of the disease is usually slow but in some seasons it is rapid. These facts make it obvious why this disease is one of the most serious problems of the cranberry growers.

It is clear that before any control measures could be advocated, more scientific knowledge was necessary concerning the nature of false blossom and the means by which it spreads. These problems have been studied by the writer. The results obtained are reported in the present paper.

## HISTORY OF DISEASE

The disease was first described and named in 1908 by Shear (21, 22). According to the best of our knowledge it originated in Wisconsin where it is believed by some growers to have been present as early as 1885 (28). It was first identified from Massachusetts in 1914 (9). At present it occurs wherever cranberries are grown commercially in the United States east of the Mississippi River. Stevens (27) and Stevens and Sawyer (28) have found by tracing sales records that the Wisconsin-grown cranberry vines



were the source of primary infection in the Massachusetts, New Jersey, and Pacific Coast bogs. Stevens and Sawyer (28) made a survey of wild bogs in Wisconsin and found false blossom on two isolated bogs. The disease was not reported on wild bogs in the New England States or Nova Scotia. The evidence accumulated by surveys of Stevens and Sawyer points strongly to the fact that false blossom is indigenous to Wisconsin.



FIGURE 1. Diseased and healthy cranberry blossoms. Note the upright manner of growth of the diseased flowers in A, in contrast with the arched healthy flowers in B.

At first the disease was attributed to faulty nutrition (14, 15, 16, 23), but a survey of bogs showed that it was present in all types of localities. In 1910 Peltier (18) made a cytological study of healthy and diseased cranberry flowers, leaves, and stems and could find no fungus or bacterium in the diseased tissues. He then suggested that it was a disease "similar to the ones causing the mosaic disease of the tobacco and the peach yellows." Franklin (9), working in Massachusetts, noted the disease in 1914 and suggested that it was probably infectious. In 1920 Fracker (8) earnestly ad-

vised that the disease be treated as though it were known to be infectious and recommended quarantine measures. A biochemical study of affected plants was made by Spaeth and Kraybill (26). They state that the disease causes a disturbance of the carbohydrate metabolism. Whether this is due to faulty nutrition or to the presence of a virus could not be determined.

Since no known parasitic organism has been found associated with this disease, since it spreads each year, and since the symptoms of diseased plants agree in many respects with those induced by known viruses, it has been considered by most workers to belong in the group of the virus diseases.

#### SYMPTOMS OF DISEASE<sup>1</sup>

The cranberry is a perennial evergreen plant with vine-like habit of growth. The side runners attain a length of three to five feet, rooting frequently and making a mat with neighboring plants. The center shoots or uprights, 12 to 18 inches high, continue growth so that the flowers appear lateral.

The false blossom disease is most readily recognized when the plant is in bloom. The name "false blossom" is particularly appropriate to the disease as the flowers show the symptoms most clearly and are usually rendered sterile. The flower, as a whole, assumes an upright position (Fig. 1, A) instead of the normal curve of the pedicel, from which the name of the plant is thought to have been derived. The arched pedicel and the flower are said to resemble the head of a crane, hence the name "crane-berry" (Fig. 1, B). The calyx lobes of diseased flowers become enlarged, the petals are short and streaked with red and green, and the stamens and pistils are more or less abnormal. When the disease is severe the entire flower may be replaced by successive whorls of leaves or by a short branch (Fig. 2).

The leaves also show symptoms of the disease. Axillary buds which are usually latent produce numerous negatively geotropic branches with many crowded leaves which are closely appressed to the stem. The resulting structure is referred to as a witches' broom. In autumn the leaves take on a reddish hue which enables one to locate a diseased area at some distance.

The cranberry sets its fruit buds for the succeeding year in autumn. In

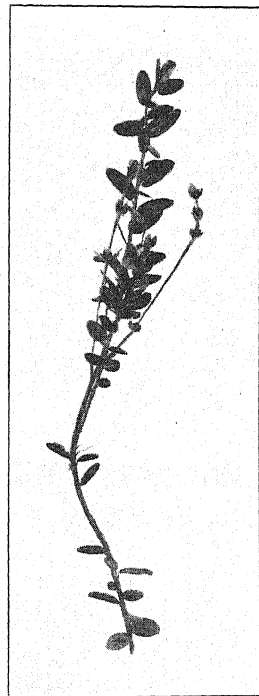


FIGURE 2. Diseased upright of cranberry showing erect pedicels. Note the three whorls of leaves as a result of floral malformation.

<sup>1</sup> The writer wishes to acknowledge the use of some unpublished morphological studies made by Dr. N. E. Stevens, which, by his permission, have been incorporated in this paper.

a diseased plant these terminal fruit buds are enlarged and in an advanced stage of development. As a result the diseased buds are frequently killed during spring frosts. One of the most striking symptoms of the disease results from the production of shoots by axillary buds which in a healthy plant would remain dormant. Cross sections were made of healthy and diseased terminal buds. A comparison of the two showed that in the diseased bud the flower rudiments are in a relatively advanced stage, and only one layer of scale leaves protects them, whereas in the small healthy bud there are at least four layers of scale leaves.

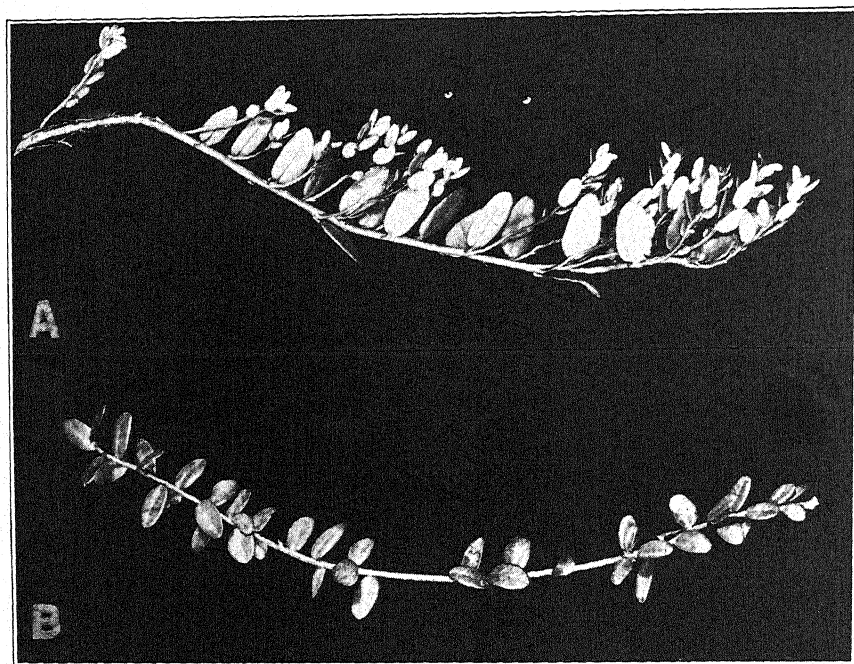


FIGURE 3. Cranberry runners. A. Diseased runner from insect-inoculated seedling of 1926. Note extreme witches' broom symptoms. B. Healthy runner from greenhouse-grown seedling.

As is well shown by illustrations published by Shear (23), flowers in various stages of phyllody are to be found on plants with false blossom. Sections of flowers which are slightly deformed show that it is the pistil which is first affected, while the stamens are apparently the last organs to disappear in the metamorphosis of the flower. Some pollen, normal in appearance, is produced in flowers in relatively advanced stages of phyllody. The condition in which seemingly normal flowers are held erect instead of drooping may well be considered the earliest stage of floral abnormality caused by false blossom. Studies on cross sections of diseased flowers show

that the petals are short, that the sepals are short and broad, that the top of the ovary is narrow, that the ovules are small, and that the stamens, though shortened and distorted, produce some pollen.

Under certain conditions diseased plants mature their fruits, but such berries are usually small, misshapen, and held in an erect position. Sections of these berries generally show only a few seeds, most of the ovules remaining undeveloped. Such seeds as are produced are generally abnormal. Some are not true seeds at all, since they apparently contain no embryo or endosperm. They consist merely of a more or less normal seed coat around a mass of tissue composed of large irregular cells containing little or no starch. Diseased seeds may contain a smaller embryo and endosperm surrounded by a thick integument. The seed has the appearance of having been arrested in its development before the embryo and endosperm had reached their full size.

The symptoms of false blossom on seedlings are more difficult to recognize. Seedlings grown in the greenhouse do not bloom before they are three years old. Though some workers are of the opinion that one must see the flower before a correct diagnosis can be made, a careful study of the leaf and twig characters has shown that these are sufficient for diagnosis. Figure 3, A shows a runner of a diseased seedling while Figure 3, B is a corresponding runner from a healthy plant. Note the numerous negatively geotropic shoots with small closely appressed leaves in Figure 3, A.

#### TRANSMISSION OF DISEASE

The field work was carried on in large measure on bogs at Toms River, New Jersey.<sup>2</sup> Some experiments and collections were made at Whitesbog and at Lakehurst, New Jersey.<sup>3</sup>

The greenhouse work was done at the Boyce Thompson Institute for Plant Research, Inc., at Yonkers, New York. To insure having healthy plants, the cranberries were grown from seed in a greenhouse. One of the greatest sources of delay was the fact that the seedlings grow very slowly. A one-year-old plant measures about four inches. Seeds of the Howes variety did not germinate so well nor so quickly as seed of the native Jerseys, the wild unselected cranberry. The best germination was obtained by placing the seeds in peat and giving them a daily alternating temperature of 20° and 30° C. They germinated in two weeks under these conditions.

#### MECHANICAL INOCULATION

Twenty unbranched, one-year-old seedlings were inoculated with juice expressed from diseased leaves. The inoculum was pricked into six differ-

<sup>2</sup> The writer is extremely grateful for the laboratory facilities, cooperation, and many kindnesses of Mr. Crabbe and Mr. Scammell of the Double Trouble Company.

<sup>3</sup> The writer wishes to thank Miss E. White and Mr. Holman for the permission to use these bogs.

ent leaves over a period of several days. The plants were kept under close observation for over a year, during which time no abnormal symptoms developed.

Grafting experiments were attempted several times. Cranberry stems are very woody, wiry, and thin. Inarching grafts were made but the plants did not grow together. No infection resulted even though the stems were in external contact for long periods of time. If the grafts had been successful infection might have resulted. The disease has not been transmitted mechanically.

#### INSECT SURVEY

It has been proved in several instances that sucking insects are the chief and most efficient carriers of certain virus diseases. The problem of the spread of cranberry false blossom was, therefore, attacked from an entomological viewpoint.

A thorough survey of the insects occurring on cranberry bogs was made. Chewing insects are very abundant and some do a great deal of damage. The Lepidoptera are extremely well represented in numbers and in species (11, 19); of the Orthoptera, Smith (25) has listed 35 species; Collembola, due to their preference for moist places, are very abundant; Coleoptera, Diptera, and Hymenoptera are represented by fewer species; among the Homoptera the cercopids and fulgorids are each represented by several species; one mealybug and one scale insect are recorded as occurring on the cranberry.

Aphids, leafhoppers, and one cercopid have been shown to be carriers of certain virus diseases. Collections of leafhoppers made in Massachusetts, Long Island, and New Jersey, and those recorded in literature are listed below; the first ten species are given in the order of their abundance on New Jersey bogs.

- |   |  |
|---|--|
| 1. <i>Euscelis striatulus</i> (Fall.)           | 13. <i>Platymetopius acutus</i> (Say)            |
| 2. <i>Platymetopius magdalenensis</i><br>Prov.  | 14. <i>Agallia 4-punctata</i> (Prov.)            |
| 3. <i>Gypona octolineata</i> (Say)              | 15. <i>Thamnotettix nigrifrons</i><br>(Forbes)   |
| 4. <i>Thamnotettix smithi</i> Van D.            | 16. <i>Chlorotettix galbanatus</i> Van D.        |
| 5. <i>Chlorotettix viridius</i> Van D.          | 17. <i>Parabolocratius viridis</i> (Uhl.)        |
| 6. <i>Cicadula sexnotata</i> (Fall.)            | 18. <i>Draeculacephala angulifera</i><br>(Walk.) |
| 7. <i>Draeculacephala mollipes</i> (Say)        | 19. <i>Thamnotettix fitchii</i> Van D.           |
| 8. <i>Agallia constricta</i> Van D.             | 20. <i>Euscelis instabilis</i> (Van D.)          |
| 9. <i>Empoasca fabae</i> (Harr.)                | 21. <i>Idiocerus provancheri</i> Van D.          |
| 10. <i>Thamnotettix melanogaster</i><br>(Prov.) | 22. <i>Acucephalus albifrons</i> (L.)            |
| 11. <i>Platymetopius frontalis</i> Van D.       | 23. <i>Platymetopius angustatus</i> Osb.         |
| 12. <i>Platymetopius hyalinus</i> Osb.          | 24. <i>Platymetopius fulvus</i> Osb.             |

- |   |   |
|---|---|
| 25. <i>Deltocephalus inimicus</i> (Say) | 35. <i>Acinopterus acuminatus</i> Van D.    |
| 26. <i>Deltocephalus micarius</i> Ball  | 36. <i>Phlepsius irroratus</i> (Say)        |
| 27. <i>Deltocephalus sandersi</i> Osb.  | 37. <i>Graphocephala coccinea</i> (Forst)   |
| 28. <i>Acurhinus pyrops</i> (Crumb.)    | 38. <i>Phlepsius tennesa</i> DeL.           |
| 29. <i>Deltocephalus osborni</i> Van D. | 39. <i>Chlorotettix unicolor</i> (Fitch)    |
| 30. <i>Euscelis cuneatus</i> S. & DeL.  | 40. <i>Chlorotettix tergatus</i> (Fitch)    |
| 31. <i>Scaphoideus</i> sp.              | 41. <i>Euscelis uhleri</i> (Ball)           |
| 32. <i>Jassus olitorius</i> Say         | 42. <i>Balclutha impicta</i> (Van D.)       |
| 33. <i>Erythroneura ziczac</i> Wlsh.    | 43. <i>Eugnathodus abdominalis</i> (Van D.) |
| 34. <i>Agallia novella</i> (Say)        |   |
44. *Chlorotettix spatulatus* O. & B.

Not all of these leafhoppers feed on the cranberry. The presence of certain weeds in the bogs determines the presence of some of the species. *Draecula-cephala mollipes*, for instance, is a grass feeder and is always found on bogs which are overgrown with grass. Such a species could probably be safely omitted from the list of suspected carriers of the disease.

Aphids were seldom found in any abundance on any bog nor were they generally present. The same was true of the species of Fulgoridae and Cercopidae which were collected at times. Working on the assumption that the carrier of false blossom was a sucking insect of wide distribution the problem became one of elimination. It might have been any of the first ten leafhoppers listed above.

#### INSECT TRANSMISSION EXPERIMENTS IN 1924 AND 1925

*Phylloscelis atra* Germ. Sixteen adults of the toad-bug, *Phylloscelis atra*, were collected on the bogs and allowed to feed for three days on diseased plants. They were then confined on a healthy plant. All of them died within a week. The plant was kept under observation for a year but no signs of the disease appeared. The experiment was not repeated in the following years as it was found that toad-bugs are not very numerous or widespread in the bogs of New Jersey. Since the toad-bug causes direct injury to the plant by sucking the juices and killing the growing tips, and since such symptoms are never associated with the disease, the insect was not tested further.

*Gypona octolineata* (Say). Several experiments were performed with the large green leafhopper, *Gypona octolineata*. Fifty of these leafhoppers were allowed to feed on diseased plants for several days. They were then transferred to five healthy seedlings where they remained from two to six weeks. The plants became yellowed and weak as a result of the feeding of these large insects, but no signs of false blossom appeared.

*Platymetopius magdalensis*, Prov. Experiments with the sharp-nosed leafhopper, *Platymetopius magdalensis*, were carried on over a period of one year. The insect has been reared on cranberry exclusively, but it has

been seen to feed on blueberry, elm, clover, and *Hypericum virginicum* L.

Eighteen experiments with this leafhopper, attempting to transfer the false blossom disease, were negative. About 500 individuals reared on diseased plants failed to transmit the disease to healthy seedlings.

#### TRANSMISSION BY *EUSCELIS STRIATULUS* (FALLEN)<sup>4</sup>

In 1926, a daily observation of the insects on the bogs was made from early June till the middle of August. It was found that during this period another leafhopper was more prevalent than the three which had already been tested. This was a light brown leafhopper with a blunt nose identified as *Euscelis striatulus* (Fallen) by Dr. Herbert Osborn.

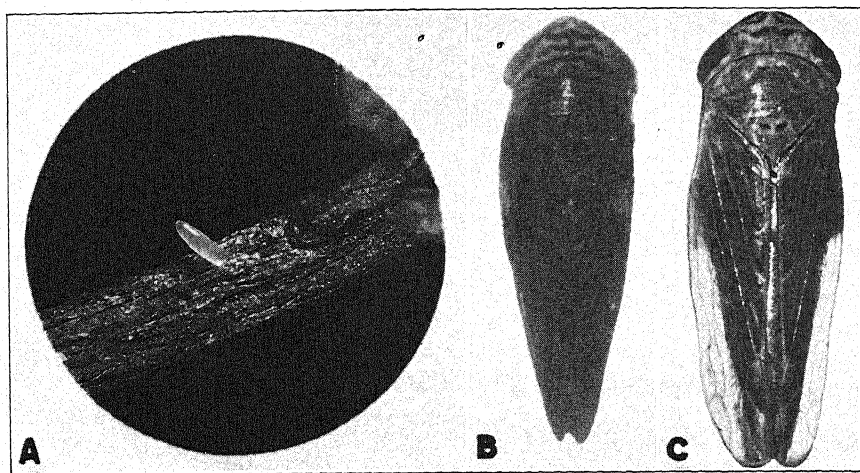


FIGURE 4. *Euscelis striatulus*. A. Egg on cranberry stem. B. Adult male. C. Adult female.

In his publication of 1890, Smith (25) said that this blunt-nosed leafhopper was the most common one on the bogs in New Jersey. Other collectors have found this to hold true for the bogs of Massachusetts and Wisconsin. In Oregon and Washington, however, this leafhopper has not been found. It is of interest to note that according to Bain (1) false blossom has not spread and is even on the decrease in this region.

Since this leafhopper proved capable of transmitting false blossom, a description of the species and notes on its distribution and life history are given here. The species was first described in Europe by Fallen in 1826. He called it *Cicada striatula* because it looked like a miniature cicada. It is such a variable species that at some time or other almost all the members

<sup>4</sup> In the most recent revision of the genus *Euscelis*, Ball (3) lists this species under the name *Ophiola striatula* (Fallen). As the new name proposed has not as yet come into general use, it is not deemed advisable to change it in this paper.

of this genus have been included under it. The egg and the adult male and female of this species are illustrated in Figure 4, A, B, and C. As Fallen's description is not easily accessible, it was thought advisable to give a technical description compiled from several sources (3, 17) and extended through the writer's own observations.

*Technical description of adult.* Sexes distinct, males dark brown, females more tawny, legs dark, femora twice annulate with pale yellow. Length of female 4.5 mm., male 4 mm., width 1 mm. (Fig. 4, B and C).

*Color:* Vertex pale yellow with three transverse bands, the posterior one broken forward on each side until it touches the middle one, its medium limb forming a crescent, the median line broadly fuscous connecting the crescent with the band in front. In dark specimens these bands become confluent and the yellow reduced to elongate spots between them. Pronotum thickly and irregularly marked with fuscous omitting an elongate spot on the anterior margin. Scutellum dark, usually the margins, a spot on apex, and a pair of elongate tri-lobed ones on disc, pale yellow. Elytra light, the inner apical cells smoky, nervures milky white, the cross nervures very broadly so, nervures broadly, heavily margined with fuscous. In dark specimens often filling up all but a small milk-white spot in the center of each cell. Face light with the sutures, arcs on front and a spot on apex of clypeus, black; or black with small spots in the middle of the facial pieces and the narrow arcs light. Below dark, anterior and middle femora with two pale yellow bands.

*Genitalia:* Female segment longer than preceding, bluntly angled and produced laterally and with medium third distinctly produced, lateral margins and pygofer pale yellow. Male valves rounded, almost semicircular, plates triangular, about twice as long as valve, a spot on each side of the disc and stout hairs on the margin, yellow.

*Life history.* The life history was worked out by field observations in New Jersey bogs, supplemented by greenhouse experiments in Yonkers, New York. The insect passes the winter in the egg stage in the cranberry plant. The egg is oval shaped, pearly white, and measures about 1.0 mm. by 0.3 mm. It is inserted longitudinally in the stem beneath the thin outer bark. Usually both ends of the egg are inserted beneath the bark leaving the center slightly exposed, but some are inserted at one end only, the remainder of the egg protruding at an angle of about 45°. This position is shown in the photograph (Fig. 4, A). The egg of this species was discovered for the first time in the autumn of 1928.

The eggs hatch in May after the water has been drawn off the bogs. Collecting with a net during the second week of June, one finds only the nymphs which are in all stages of development. There are five instars in the nymphal life. The first adults are collected in the third week of June and are all males. The females do not mature until one or two weeks later than the males. The sexes can be distinguished even as nymphs by the fact that females are light yellowish in color and males are olive green. The adults are readily separated as to sex because the female is always larger and of a lighter brown color than the male.

Judging from the fact that almost no nymphs are found after July, it is believed that there is only one generation a year. Adults collected as late



as September and October are almost invariably females. They lay their eggs over a long period of time, since the eggs hatch over a period of a month or longer.

*Habits.* This insect is most abundant during the hottest months, July and August. It is most active during the sunniest and warmest part of the day. When disturbed, the insect flies close to the vines, covering a distance of two or three feet at the most, and then settles down on the stem of another plant. It alights usually with head upward and legs clasping the stem; only rarely does it alight on a leaf. In feeding it is usually found on the stem near the base of a leaf. When confined to plants in cages it seldom moves from its characteristic position along the stem. The females lay very few eggs when kept in cages.

On a badly diseased bog at Lakehurst, New Jersey, this species was exceedingly numerous. In 25 sweeps of a net an average of 100 of these leafhoppers was caught. The insects were much less numerous on healthy bogs. Why this species should occur more abundantly in diseased than in healthy bogs is a subject for speculation. Is the disease more abundant because the insects are more numerous or vice versa? Is the diseased plant more agreeable to the insect because of its higher carbohydrate content? Possibly the location of certain bogs may be especially favorable to the insect. In newly planted bogs where the plants are distinct from each other the spread of disease is more rapid and complete than in old bogs (2).

*Cytology.* Since *E. striatulus* was found to be the carrier of cranberry false blossom, and since no known causative organism has been found in the plant, it was decided to make a cytological study of the insect to see if the etiological agent could be found.

No inclusions or x-bodies have been found in the cells of diseased plants. A histological and cytological study of the leafhopper was made in the hope of finding some abnormality, such as the enlargement of some organs or cells, cysts in the stomach wall, lesions in the salivary glands, or inclusion bodies in certain cells, such as are associated with certain animal virus diseases.

A study of this nature had already been made by the author (7) on the leafhopper *Cicadula sexnotata* (Fallen), the carrier of the aster yellows virus. The technique and staining in the present work were the same as that employed in the above mentioned paper.

Paraffin sections of over 100 adults of *E. striatulus* were made and colored with Giemsa stain. This stain is suitable for the demonstration of any bacteria or rickettsia. The morphology of this leafhopper closely resembles that of *Cicadula sexnotata*. A detailed study of the tissues was made to see if they harbored any visible parasites or symbionts, and to see if there was any difference between healthy and viruliferous leafhoppers.

As is well known the large American roach, *Periplaneta americana* L., has rickettsia-like bodies which are located in special cells known as bacteriocytes. Glaser (12, 13) has cultured these organisms and considers them to be bacteria. The wall of the abdomen of *Euscelis striatulus* is lined with a thick layer of fat cells called adipose tissue. This tissue was carefully examined for rickettsia-like bodies but none were found.

The malpighian tubules are four in number and fill the lower half of the abdomen (Fig. 5, A). The lumen of these was often found to be filled with a clear pink-staining secretion. Cowdry (5) reports finding rickettsia in the cells of the tubules of several species of insects. No organisms of any kind were found in the tubules of this leafhopper.

The muscles which are especially large in the thorax stain very well. The nuclei are small and elongate following the course of the muscle fibers. If any parasites were present in this tissue they should be readily visible. None could be found.

The nervous system is rather large, occupying a considerable portion of the head and having several large ganglia concentrated in the thorax. The structure of the cells and fibers is sharply brought out by the stain. No foreign organism was found in this system.

The mycetome is a hereditary organ, composed of an aggregate of cells containing organisms of a fungal nature. This has been figured and described by the author (7) for *Cicadula sexnotata*. Since this organ is present in virus-free as well as virus-bearing insects, it is not believed to be associated with disease transmission.

More attention was concentrated on the salivary glands than on any of the preceding organs because they have been more often reported as the seat of parasitism. The salivary glands of this species resemble those of *Cicadula sexnotata* so closely that it is difficult to tell them apart. In arrangement, size, and types of acini they agree closely. Each acinus is binucleate as can be seen in Figure 5, C and D. Physiologically there are three types of acini: the mucous, the serous, and those lining the accessory or reservoir glands. They all stain differently due to their structure and no doubt also to a difference in acidity (Fig. 5, D). No foreign organism was found in the glands.

The alimentary tract is the most logical place to look for parasites and foreign organisms, since they are usually ingested. As the intima of the fore- and hind-intestine is shed several times during the life of the insect, the mid-intestine which is not lined with intima would be the place to look for a parasite. In three of the 103 insects studied numerous bacteria were found scattered with the food in the lumen of the mid-intestine. None of the bacteria were intracellular, therefore they must have been ingested. Figure 5, E shows a mass of bacteria which has just passed through the oesophageal valve. Other bacteria were found above and below the valve.

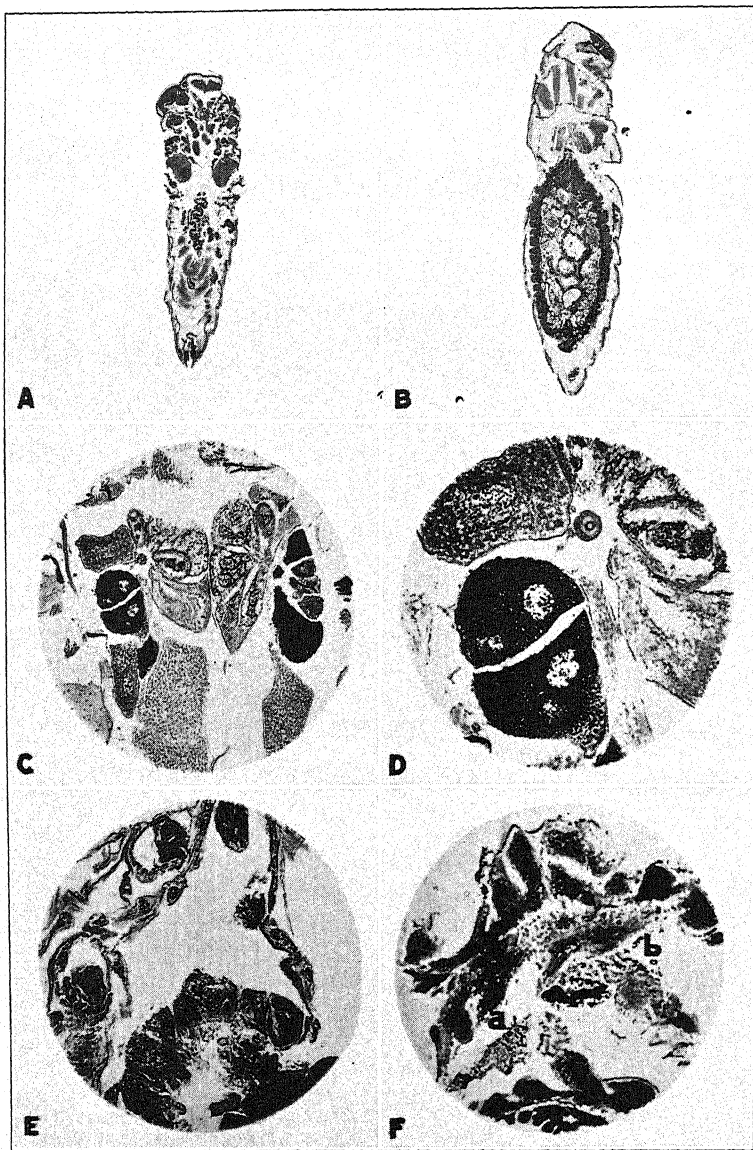


FIGURE 5. Cytological studies of *Euscelis striatulus*. A. Longitudinal section of normal leafhopper. B. Longitudinal section of leafhopper parasitized by a pipunculid larva. The parasite fills the entire abdomen of the host. C. Longitudinal section of the salivary glands. D. Enlargement of a few acini of C. Note the circular cross section of the salivary duct. E. Mid-intestine showing oesophageal valve at top and a mass of bacteria below and to the right of it. F. Enlargement of a portion of the mid-intestine showing cells with large nuclei, and scattered bacteria in the two food masses *a* and *b*.

Figure 5, F shows large stomach cells and bacteria in the food (a and b). As all these insects were captured in the field, no significance is attached to the presence of bacteria in these three individuals.

A study of the stomach or mid-intestinal cells and their nuclei was made to find if they contained any inclusion bodies or rickettsia. Each cell contains two large nuclei with granular scattered chromatin. With certain fixatives and stains these chromatin granules appear very regular and might be mistaken for rickettsia if one were not aware of the fact that each cell contains *two* nuclei.

A comparative study of healthy and viruliferous insects in regard to the size, structure, and appearance of the different organs gave no evidence of any difference between the two.

#### EXPERIMENTAL WORK OF 1926

The experiments carried on during the summer of 1926 with *Euscelis striatulus* gave the first evidence that this is the insect that transmits cranberry false blossom (6). The insects were collected from bogs in which the disease was prevalent, and then fed on infected plants for a period of two weeks, to insure the presence of the virus in the insects. The seedlings used, about 1,500 in all, had been grown under controlled greenhouse conditions in Yonkers. They were taken to New Jersey and exposed in ten lots to a strong culture of viruliferous leafhoppers for a period of at least two weeks. Figure 6 shows a lantern globe used in the inoculation studies. After the insects had been taken off the plants were set out in a nursery where they remained during that fall and winter. On July 10, 1927, these seedlings were moved to the experimental bog (Fig. 7) which adjoins an infected commercial bog. During the course of that summer it was observed that approximately 90 per cent of the exposed seedlings showed definite symptoms of the disease. About 500 seedlings which had been reserved as checks inadvertently became diseased due to exposure to disease-carrying insects. Since the spread of the disease in the plants in the adjacent area was observed to be considerably less than in the experimental plot, the writer felt that there was little doubt that *Euscelis striatulus* was the carrier of false blossom.

#### EXPERIMENTAL WORK OF 1927

During the summer of 1927, the writer was not able to continue the work in person. Through the cooperation of Dr. N. E. Stevens, some field experiments were carried on by Mr. C. O. Bratley. Six plots three feet square were laid out in a commercial bog relatively free of the disease. Boards were sunk into the soil, and cheese-cloth cages were erected over the enclosed vines. Into these cages numbers of the species *Euscelis striatulus* collected on diseased bogs were placed. Other cages had a mix-

ture of several species of leafhoppers. The number of diseased and healthy upright branches in each cage was counted before the insects were put in. In 1928 this area was kept under water until July so no observations were made

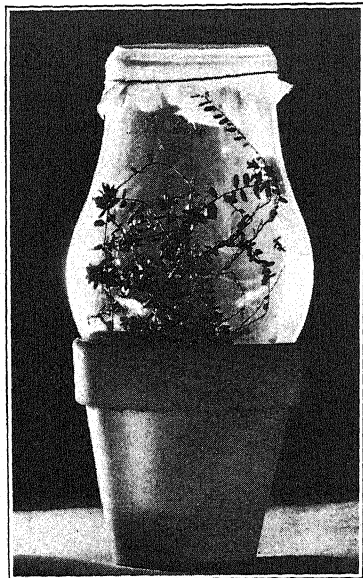


FIGURE 6. Lantern globe cage used for insect inoculations in all seedling experiments.

on this experiment until the following year. In 1929 these six plots were examined and all were found to contain more diseased uprights than were to be found in surrounding areas of equal size. This same type of experiment was carried out at Whitesbog, New Jersey, by Beckwith and Hutton (4) during the season of 1927. They found an increase of 15 times the number of diseased uprights in the area on which *Euscelis striatulus* was confined. Dr. Franklin, working at Wareham, Massachusetts, also tested out a mixed population of leafhoppers on enclosed vines and got an increase in the number of diseased uprights. All of these experiments gave evidence of transmission of false blossom by *Euscelis striatulus*. They did not, however, give satisfactory proof because the plants used were not known to be healthy at the time they were exposed to the blunt-nosed leafhopper, and because insect species other than *E. striatulus* were present in the caged areas. Experiments

that were more carefully controlled were necessary to bring satisfactory proof of the relation of *E. striatulus* to the false blossom disease.

#### EXPERIMENTAL WORK OF 1928

More than 500 individuals of *Euscelis striatulus* were collected at Toms River and Whitesbog in early July. These were brought to the laboratory at Yonkers, and there allowed to feed on diseased plants for a week before being placed on healthy one-year-old seedlings. (Table I.)

These seedlings were kept in the greenhouse during the winter and examined frequently. By April 3, 1929, some of the seedlings in experiment 15 produced excessive shoots, the first symptoms of the disease. On May 31, 1929, one of the seedlings of this lot was markedly diseased. The photograph (Fig. 8, A) made of this plant on June 17, shows six small negatively geotropic shoots growing from the tip of the runner. This same plant is shown in Figure 8, B, photographed on October 10, to show the further growth of these shoots which form a sort of witches' broom. Figure 9 shows four seedlings from this experiment with a check plant to the right

of each. Note the upright growth habit of the diseased seedlings, the smaller more delicate leaves, and the more profuse branching.



FIGURE 7. View of commercial bog at Toms River, New Jersey, with experimental plot in the foreground. Seedlings inoculated in 1928 are in the cage.

TABLE I  
INSECT TRANSMISSION EXPERIMENTS IN 1928 WITH *EUSCELIS STRIATULUS*

Exp. No.	No. of plants	No. of leaf-hoppers	Inoculation period in days	Observations 1930		
				Diseased	Healthy	Dead
1	15	20	15 (July 18-Aug. 1)	—	15	—
2	15	20	15 " " " "	4	—	11
3	14	20	15 " " " "	—	14	—
4	15	20	15 " " " "	12	—	3
5	15	30	15 " " " "	—	14	1
6	15	20	14 (July 19-Aug. 1)	12	—	3
7	15	20	14 " " " "	14	—	1
8	15	20	14 " " " "	2	5	8
9	15	20	14 " " " "	3	6	6
10	15	20	14 " " " "	11	—	4
11	15	20	14 " " " "	6	—	9
12	15	20	14 " " " "	3	—	12
13	15	32	14 " " " "	5	1	9
14	15	20	14 " " " "	—	14	1
15	15	20	14 " " " "	7	1	7
16	15	20	14 " " " "	12	—	3
17	15	20	15 (July 18-Aug. 1)	5	—	10
18	15	20	15 " " " "	2	9	4
19	1	50	25 (July 18-Aug. 16)	1	—	—
20	15	20	2 (July 18-July 20)	2	2	11

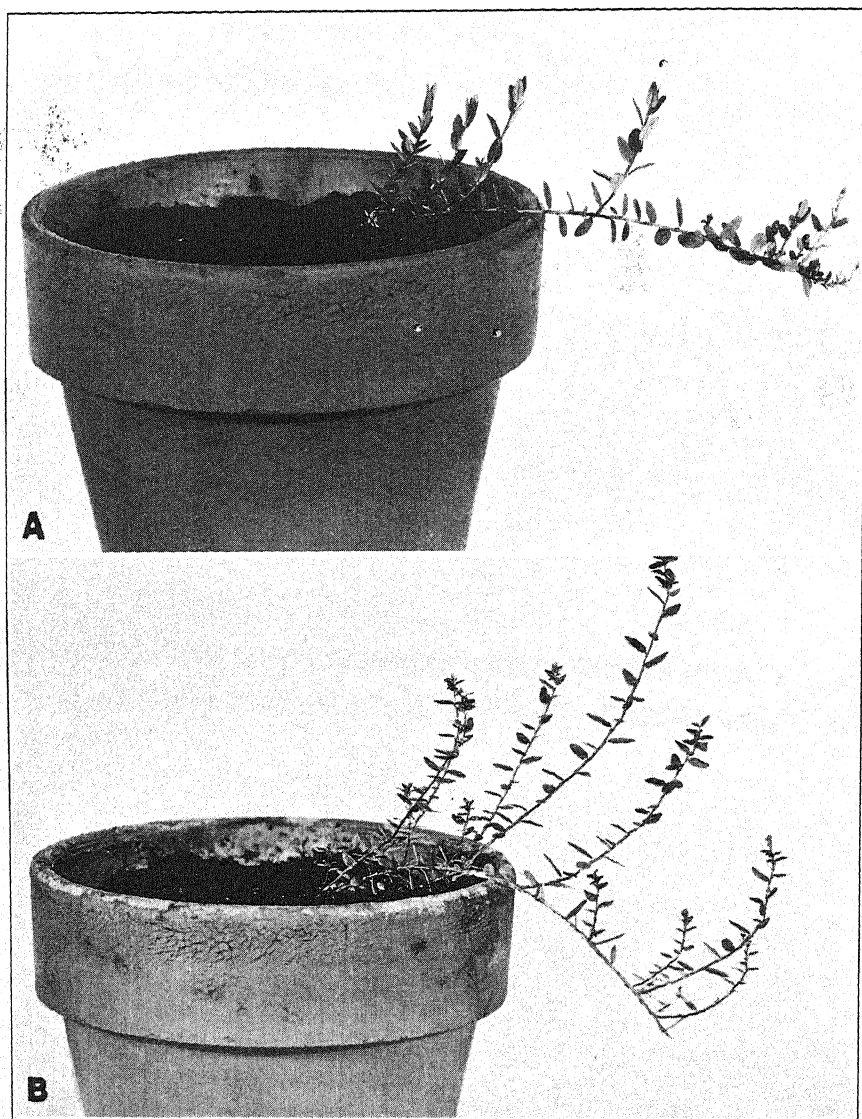


FIGURE 8. Diseased cranberry seedlings. A. One-year-old seedling showing early symptoms of false blossom. B. Same seedling four months later. Note extreme witches' broom symptoms.

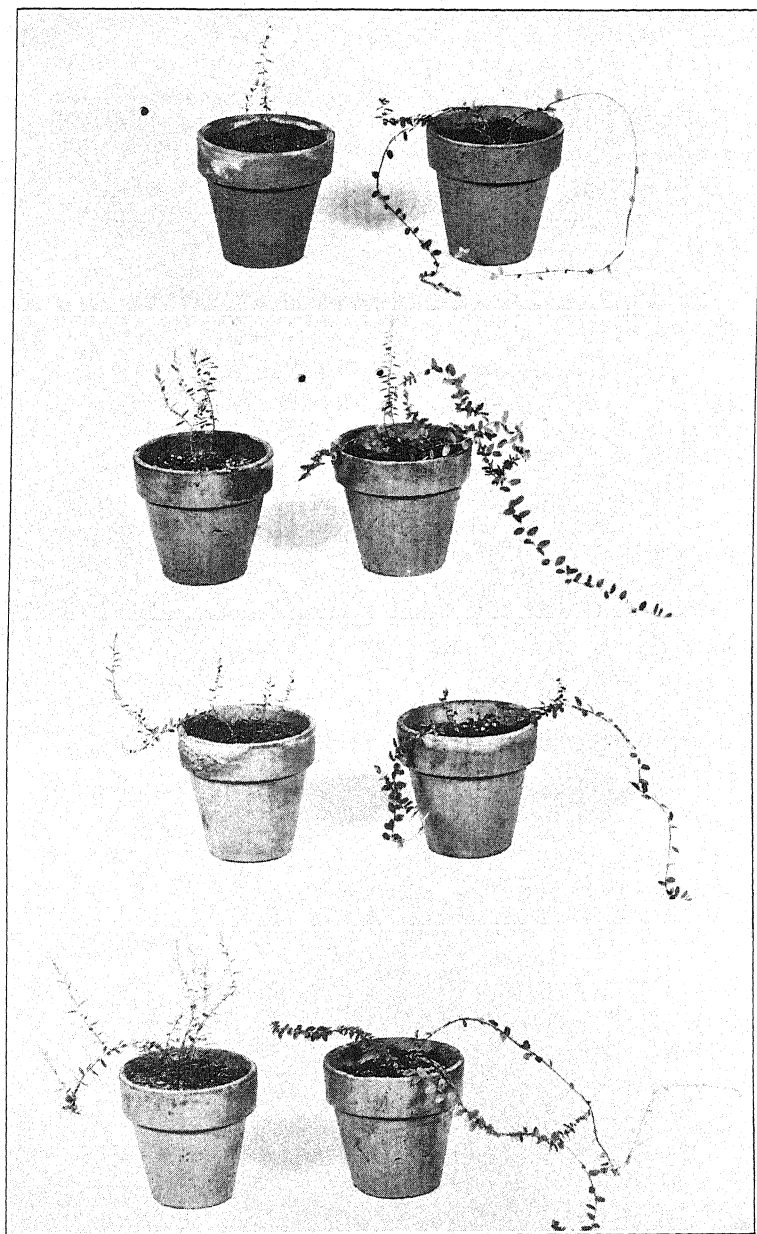


FIGURE 9. Series of insect-inoculated cranberry seedlings of 1928. Check plant to the right of diseased plant in each case. Seedlings are one year old.



Experiment 19 consists of a single three-year-old seedling on which 50 disease-bearing leafhoppers had been caged. On May 14, 1929, four tips on this plant began to produce numerous shoots from normally latent adventitious buds. But more striking still was the formation of two flower buds which failed to produce normal flowers. Figure 10 shows the typical upright manner of growth of these two "false blossoms." They never developed petals. As the photograph shows, leaves grew from the center of the larger bud. This is the same type of symptom as that illustrated by Shear (23).

During the summer of 1928, carefully controlled field experiments were carried out at the Double Trouble bogs in New Jersey with the cooperation

of Dr. R. B. Wilcox and Mr. Scott. Healthy seedlings reared in Yonkers were taken to the New Jersey laboratory. One hundred and twenty seedlings were exposed to 100 disease-carrying leafhoppers for two weeks. Sixty seedlings were kept caged and free of all insects, to serve as checks. All of these seedlings were put out in the experimental bog that autumn and caged (Fig. 7). About one-half of the exposed seedlings survived the winter. In the summer of 1929 all of the plants that had been exposed to *E. striatulus* were diseased. All of the 60 check plants remained healthy and made a luxuriant growth.

#### EXPERIMENTAL WORK OF 1929

In order to find out what insects wintered over in the cranberry plant or its substrate, about four square feet of sod with diseased vines was brought into the greenhouse from Whitesbog on April 24. The water had been drawn



FIGURE 10. Fruiting upright taken from three-year-old cranberry plant with two false blossom buds. Note the leaf growing from the center of the upper bud.

off the bog the preceding week. These plants were placed in a cage in a warm greenhouse. By June 12 the following adult insects had been reared from the plants and the soil:

Homoptera (leafhoppers)

50 *Euscelis striatulus*

7 *Platymetopius magdalensis*

6 *Gypona octolineata*

1 *Deltocephalus* sp.

Other individuals in the Collembola, Coleoptera, Lepidoptera, Diptera, and Hymenoptera were found.

Forty-six adults of *E. striatulus* reared in this cage on the diseased vines were used in an inoculation experiment. They were placed on two healthy seedlings on June 18 and taken off July 16. By July 22 these two seedlings had sent out numerous small axillary shoots. Figure 11 shows the plants as they looked on August 23. This experiment shows that under favorable conditions the symptoms of the disease may appear during the same season that inoculation occurred. In the case cited, the period between initial inoculation and appearance of symptoms was about a month.

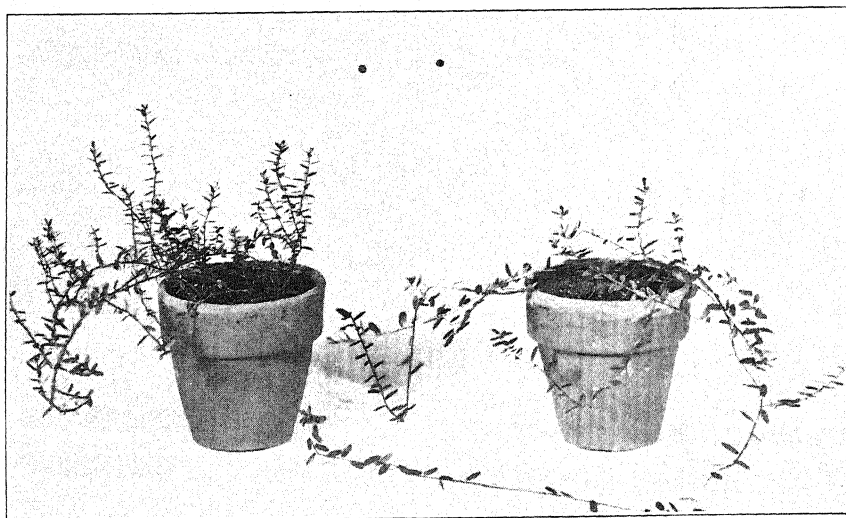


FIGURE 11. Two-year-old cranberry seedlings of 1929 insect-inoculated. Check seedling to the right of diseased seedling which showed symptoms of false blossom one month after initial inoculation.

At the same time experiments were made with the two leafhoppers, *Platymetopius magdalensis* and *Gypona octolineata*, from the same source. All seedlings exposed to these species remained healthy.

On June 19 and 20, more than 1000 nymphs and adults of *E. striatulus* were collected on some severely diseased bogs at Lakehurst. These insects were used in several series of experiments through the summer. A record of the first two experiments is shown in Table II.

Table II shows that few of the 52 plants used in experiments 21 and 22 became diseased. Yet when these same insects were taken off on July 16 and put on other healthy seedlings they produced almost 100 per cent disease. The writer is inclined to believe that there is some evidence here of an incubation period of the virus in the insect. Many of the insects used were in the late nymphal stages.

TABLE II  
EXPERIMENTS WITH VIRULIFEROUS LEAFHOPPERS IN 1929 WITH *EUSCELIS STRIATULUS*

Exp. No.	No. of plants	No. insects per pot	Inoculation period in days	Observations 1930		
				Diseased	Healthy	Dead
21	28 2 per pot	50	22 (June 25-July 16)	3	9	15
22	24 2 per pot	50	21 (June 26-July 16)	2	16	6

The insects from experiments 21 were used in experiment 24 A-K and those of experiment 22 in experiment 23 A-B. The results are as shown in Table III.

TABLE III \*  
EXPERIMENTS WITH VIRULIFEROUS LEAFHOPPERS (*EUSCELIS STRIATULUS*)\*

Exp. No.	No. of plants	No. of insects	Inoculation period in days (Begun July 16)	Observations 1930		
				Diseased	Healthy	Dead
23 A	6	240	15	3		3
23 B	6	400	15	5		1
24 A	2	50	17	1		1
24 B	2	50	16	1		1
24 C	2	50	17	2		
24 D	2	50	16	2		
24 E	2	50	14		2	
24 F	2	50	7	2		
24 G	2	50	17	2		
24 H	2	50	15	2		
24 I	2	50	17	2		
24 J	2	50	7	2		
24 K	2	50	14		1	1

\* The leafhoppers used in the above experiments were the same individuals as used in Experiments 21 and 22.

TABLE IV  
EXPERIMENTS WITH VIRULIFEROUS LEAFHOPPERS (*EUSCELIS STRIATULUS*)\*

Exp. No.	No. of plants	No. insects for 2 plants	Inoculation period in days	Observations 1930		
				Diseased	Healthy	Dead
25	2	20	25	2		
26	2	40	28	2		
27	2	15	28	2		
28	12	50	21	12		
29	2	35	20	2		
30	2	40	28	1		1
31	2	40	27	2		
32	2	26	27	2		
33	2	24	23	2		
34	2	40	23	2		
35	10	50	23	8	1	1
36	12	50	21	7	4	1
37	6	40	20	4	2	

\* The leafhoppers used in the above experiments were the same individuals as used in Experiments 23 and 24.

The leafhoppers used in the experiments listed in Table III were later used in successive transmission experiments from July 30 to September 10, 1929. The results of these experiments are recorded in Table IV. By the end of September only a few insects were left and of these all were females. They all died during the following month.

Hundreds of check plants kept under the same greenhouse conditions as the plants in Tables III and IV but not exposed to leafhoppers remained healthy.

#### EXPERIMENTS WITH VIRUS-FREE LEAFHOPPERS

An effort was made to determine whether leafhoppers which had not fed on diseased vines could cause the disease. On July 15 several hundred hoppers were collected on a bog in Toms River in which there was no evidence of disease. These insects were put directly on healthy seedlings with results as shown in Table V.

TABLE V  
EXPERIMENTS WITH HEALTHY LEAFHOPPERS

Exp. No.	No. of plants	No. of insects	Inoculation period in days	Observations 1930		
				Diseased	Healthy	Dead
38	2	300	8 (July 15-22)	1?	2	
39	2	50	29 (July 22-Aug. 19)		1	
40	2	84	10 (July 22-31)		1	1
41	2	50	20 (July 31-Aug. 19)		1	1
42	2	28	20 (July 31-Aug. 19)		2	
43	2	52	23 (Aug. 19-Sept. 10)		1	1

A further experiment was made with leafhoppers including *Euscelus striatulus* collected on some old bogs at Manorsville, Long Island, New York. False blossom has never been reported from Long Island. These insects were used on 16 seedlings. Twenty-five hoppers to each two seedlings were kept on the plants from August 5 to August 26. At the present time (April 15, 1930) all of these plants appear to be healthy. Check plants not exposed to insects remained healthy. Leafhoppers which have not previously fed on diseased cranberry plants, cannot transmit the disease.

Tables III and IV show that almost 90 per cent of the surviving plants became diseased. The failure to transmit the disease in some cases may be due to the fact that many different factors are involved. The age of plant and the conditions under which it grows are doubtless factors in the problem of disease transmission. The writer's experiments indicate that the plant must be growing rapidly at the time of exposure if high percentages of transmission are to be obtained. It has been shown that the leafhoppers which transmit the virus of aster yellows, maize streak, and curly top of sugar beets do not transmit the disease immediately after feeding on diseased plants. A few hours to several days, as the case may be, must elapse

in order for these leafhoppers to become viruliferous. This same condition may obtain in the case of the cranberry leafhopper. This is a point which will receive more attention in future work on the problem.

Other insects may possibly be concerned in the spread of false blossom disease. No evidence of the spread of the disease by any other insect has yet been obtained. In the case of curly top of sugar beets there seem to be two leafhoppers, *Agallia sticticollis* Stål and *Eutettix tenellus* (Baker), which can transmit the virus of this disease.

#### OTHER HOST PLANTS OF FALSE BLOSSOM

A search for false blossom on bog plants other than the cranberry was made each year. None of the plants bordering diseased bogs were found to show symptoms. Surveys made on wild bogs by Sawyer (unpublished work) gave us the information that *Vaccinium oxycoccus* L., the small European cranberry, is also susceptible to the disease.

Attempts made by the writer and other workers to transmit false blossom to the blueberry, *Vaccinium corymbosum* L., were unsuccessful. The insects died in three days when caged on this plant.

#### CONTROL MEASURES

It is evident that false blossom could be controlled if the insect that transmits it were held in check. A large number of individuals of *Euscelis striatulus* have been observed to be parasitized by a species of dryinid and by a species of pipunculid. Figure 5, B shows a longitudinal section of a pipunculid-parasitized leafhopper. The parasite fills the entire abdomen of the host after having eaten all the organs except the alimentary tract. The leafhopper dies when the parasite leaves it. There is no external evidence of the presence of the pipunculid parasite as there is in the case of the dryinid. The latter is seen as a small grayish sac attached to the abdomen of its host. This parasite also causes the death of its host. It was noticed that there was a significant difference in the greater number of females parasitized as against the males. These parasites are one of the natural control measures of this leafhopper.

One of the chief methods of insect control on wet bogs, that is, bogs which have a reservoir, has been to flood the bogs for a long or short period, as the case may demand. For the control of certain insects the bog is submerged for 24 hours after which the water is drawn off. To insure the destruction of all the insects the trash and bordering plants are burned with kerosene oil. When a bog is badly infested, the grower holds the winter water flow on the bogs until July, thereby killing all insects at the cost of that season's crop.

Due to the new information on the relationship between the leafhopper and false blossom, the cranberry growers have been taking vigorous meas-

ures to control leafhoppers in regions where the disease is spreading rapidly. Some growers have kept their bogs flooded until July. Others have flooded them for 12 hours in June just before the flower buds open and after the leafhopper eggs have hatched. During the summer of 1929 a spraying campaign against insects was carried out in New Jersey (4). Pyrethrol, a proprietary pyrethrum-soap mixture, was found to be effective against the leafhoppers. Sweeping with a net before and then a few hours after spraying showed a 90 per cent reduction in the numbers of leafhoppers.

If a bog is badly diseased the only effective measure of control is to burn or turf the old vines and replant. The fact that some varieties of cranberry are more resistant than others to false blossom points the way for future progress. The McFarlin variety has been shown by Fracker (8) to be the most resistant. Many growers are attempting to secure this variety for their new plantings. A search is being made in old diseased bogs for such plants as are still healthy in order to find more resistant varieties.

#### SUMMARY

1. Cranberry false blossom is a virus disease which has become a serious problem in the culture of the cranberry. The disease is considered to be indigenous to Wisconsin, as it was found there on wild bogs. It is characterized by malformation of the flower, stunting of the plant, and the production of witches' broom symptoms.
2. Attempts at mechanical transmission of the disease were unsuccessful.
3. A list of 44 species of leafhoppers found on cranberry bogs collected in an insect survey is given.
4. Insect experiments to transmit the disease with *Phylloscelis atra* Germ., the toad-bug, *Gypona octolineata* (Say), the large green leafhopper, and *Platymetopius magdalenensis* Prov., the sharp-nosed leafhopper, were unsuccessful.
5. The first successful transmission of false blossom was made in 1926 with the leafhopper *Euscelis striatulus* (Fallen). Successful transmission of the disease in field experiments in 1926 and 1927 were corroborated by laboratory experiments in 1928 and 1929.
6. A description and an account of the life history and habits of the insect vector are given.
7. A cytological study of the carrier, *Euscelis striatulus*, revealed no differences between healthy and viruliferous insects. Bacteria ingested with the food were found in 3 out of 103 insects.
8. *Euscelis striatulus* adults collected on apparently healthy bogs did not transmit the disease.
9. Several hundred check plants kept in the greenhouse remained healthy.

10. Transmission experiments with viruliferous individuals of *Euscelis striatulus* on *Vaccinium corymbosum* L. failed.

11. *Euscelis striatulus* is parasitized by a species of dryinid and a species of pipunculid.

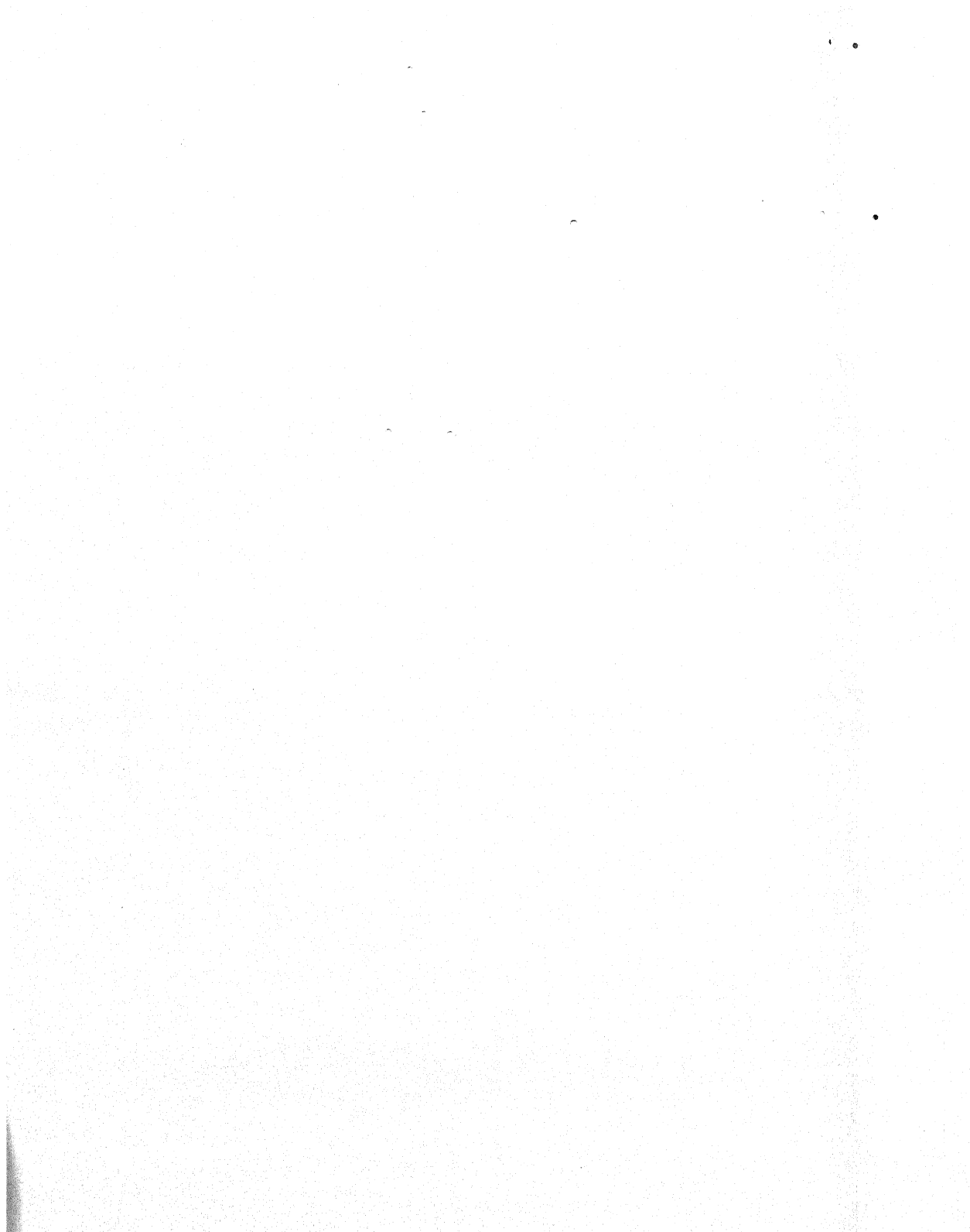
12. It is suggested that to control false blossom measures be taken to reduce the numbers of the insect vector on bogs and that a search be made for resistant varieties of the cranberry.

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# STUDIES ON ASTER YELLOWS IN SOME NEW HOST PLANTS

L. O. KUNKEL

## INTRODUCTION

Most of the virus diseases of plants are known to attack only a small number of species. A few have been shown to have rather wide host ranges. Wingard (14) transmitted the ring spot disease of tobacco (*Nicotiana tabacum* L.) to 38 genera belonging in 17 different families of plants. The curly top disease of beets (*Beta vulgaris* L.) has also been transmitted to a rather large number of species. Its hosts include plants belonging in some 15 different families (1, 8, 10). Further studies will probably show that many other virus diseases have wider host ranges than have been recognized.

In a former paper (4) the writer recorded the experimental transmission of aster yellows to more than 50 species of plants belonging in 23 different families. During the past four years he has made further studies on the host range of the disease. Yellows has been experimentally transmitted to 120 species of plants not previously recognized as hosts of the disease. The object of this paper is to report the results of these further transmission experiments and to describe briefly the symptoms of aster yellows on the different hosts in which it has been studied.

## METHODS

The methods employed in the transmission of aster yellows to the hosts here reported were similar to those described in the paper to which reference has already been made (4). Cultures of the insect vector, *Cicadula sexnotata* (Fall.), were maintained continuously in insect-proof cages in a greenhouse. Most of the virus-free cultures were kept on young rye (*Secale cereale* L.) plants. Virus-bearing cultures were kept on yellowed aster (*Callistephus chinensis* Nees.) plants. The insects flourished on these plants and produced a new generation every 30 to 50 days depending on the temperature at which they were held. The life cycle is shorter at temperatures slightly above 80° F. than at lower temperatures.

Attempts were made to grow the insect on various fruits and vegetables. This was done in the hope of finding a method of maintaining colonies without having to supply them with growing plants. The following were used: apple, pear, banana, potato, sweet potato, green tomato, parsnip, carrot, white turnip, and yellow turnip. Of all the fruits and vegetables tested the yellow turnip or rutabaga proved most suitable. Small blocks of raw yellow turnip were placed in liter flasks together with colonies of the leafhopper. The tops of the flasks were fitted with cloth covers. The leafhoppers fed freely on the turnips and in due time the females deposited

eggs in the blocks. Nymphs hatched from these eggs were reared to maturity on the raw turnip blocks. Adults thus obtained deposited eggs from which a second generation was reared. Although it was possible for the insect to complete its life cycle and to breed and flourish on the turnip blocks, this method of maintaining cultures did not prove to be satisfactory. The chief difficulty was with fungi and bacteria which caused the blocks to rot after varying periods of time. The method proved to be more difficult than that of maintaining colonies on living plants. It was, therefore, abandoned. The experiment is reported here because it proves the ability of the insect to live on a medium other than green plants.

Each species tested for susceptibility to aster yellows was grown from seed in a greenhouse. When the seedlings reached a proper size they were exposed to virus-bearing colonies of leafhoppers for a period of one to two weeks. From four to eight seedlings of each species were thus exposed. Four or more healthy young aster plants were also exposed at the same time but usually for a shorter period than the test plants. The insects were always forced to feed at least two days on the plants being tested. Two or more seedlings of each species were kept free from insects and served as checks. Nymphs hatched from eggs deposited in the plants while they were being exposed to the insect colonies were killed by fumigating with hydrocyanic acid gas. If the aster plants and one or more of the exposed seedlings developed symptoms characteristic of aster yellows while the check seedlings remained healthy, the disease was considered to have been transmitted to the species in question. In some instances it was transmitted from the test plants back to the aster. While such transmission is necessary in order to bring full proof that the disease observed on any yellowed plant is actually aster yellows, the symptoms are so similar on the different host plants that this step was not considered necessary in most cases. If none of the exposed test plants of a given species developed symptoms of aster yellows after a period of five or six months, the species was considered to be immune or highly resistant. The number of species that proved to be susceptible to yellows was only a small part of the number exposed to virus-bearing insects.

In all instances except one, transmission was affected by means of *Cicadula sexnotata*. Aster yellows was not taken to the tomato except by the transplantation of buds from yellowed *Nicotiana rustica* L. plants.

It has been found that the symptoms of aster yellows on different host plants vary considerably with the age of a plant at the time it becomes infected. In order to obtain a complete picture of the disease in any given host it is necessary to observe it in plants that have been infected at different stages in their development. The symptoms shown by plants that become infected while young are often very different from those shown by plants which take the disease as they near maturity. Such observations

have not been made for many of the hosts here reported. Symptoms have been described in most instances from a study of plants that took the disease while young. Under such circumstances all but highly tolerant hosts fail to produce flowers. For this reason the effect of the disease on the flowers of many of the species studied has not been observed.

#### SUSCEPTIBLE PLANTS

The new hosts to which aster yellows was transmitted or from which aster yellows was carried to healthy aster plants are shown in Table I. The plus signs indicate the plants from which and to which the disease was transmitted. Pictures of diseased and healthy plants or parts of such plants are used to show the effects of aster yellows on a number of the new host species. A brief description of the diseased plants of each species listed in Table I is given below.

*Humulus japonicus* Sieb. et Zucc. Plants are chlorotic and produce long slender secondary shoots.

*Monolepis chenopodioides* (Nutt.) Moq. Plants are badly dwarfed and chlorotic. They produce narrow leaves borne on long upright-growing petioles. Many secondary shoots are produced as is shown in Figure 20.

*Abronia umbellata* Lam. var. *grandiflora*. Plants are chlorotic. Many slender secondary shoots are produced.

*Tetragonia expansa* Murr. Plants are dwarfed and chlorotic. They grow small narrow leaves and numerous slender secondary shoots. A healthy and a yellowed branch are shown in Figure 14.

*Dianthus alpinus* L. Plants are slightly chlorotic. They produce small leaves and an abnormal number of secondary shoots as is shown in Figure 13. Plants that have had the disease for some time produce extremely small leaves.

*Herniaria glabra* L. Plants produce many secondary shoots. They do not blossom and are slightly chlorotic.

*Lychnis coronaria* Desr. Plants are much dwarfed and slightly chlorotic. Many secondary shoots are produced.

*Lychnis viscaria* L. Plants are slightly chlorotic and are dwarfed as is shown in Figure 35.

*Polycarpon tetraphyllum* L. Plants are slightly chlorotic. Numerous secondary shoots are produced. The leaves are elongated and much narrower than the leaves of healthy plants.

*Tunica saxifraga* (L.) Scop. Plants are dwarfed and slightly chlorotic. They produce large numbers of secondary shoots.

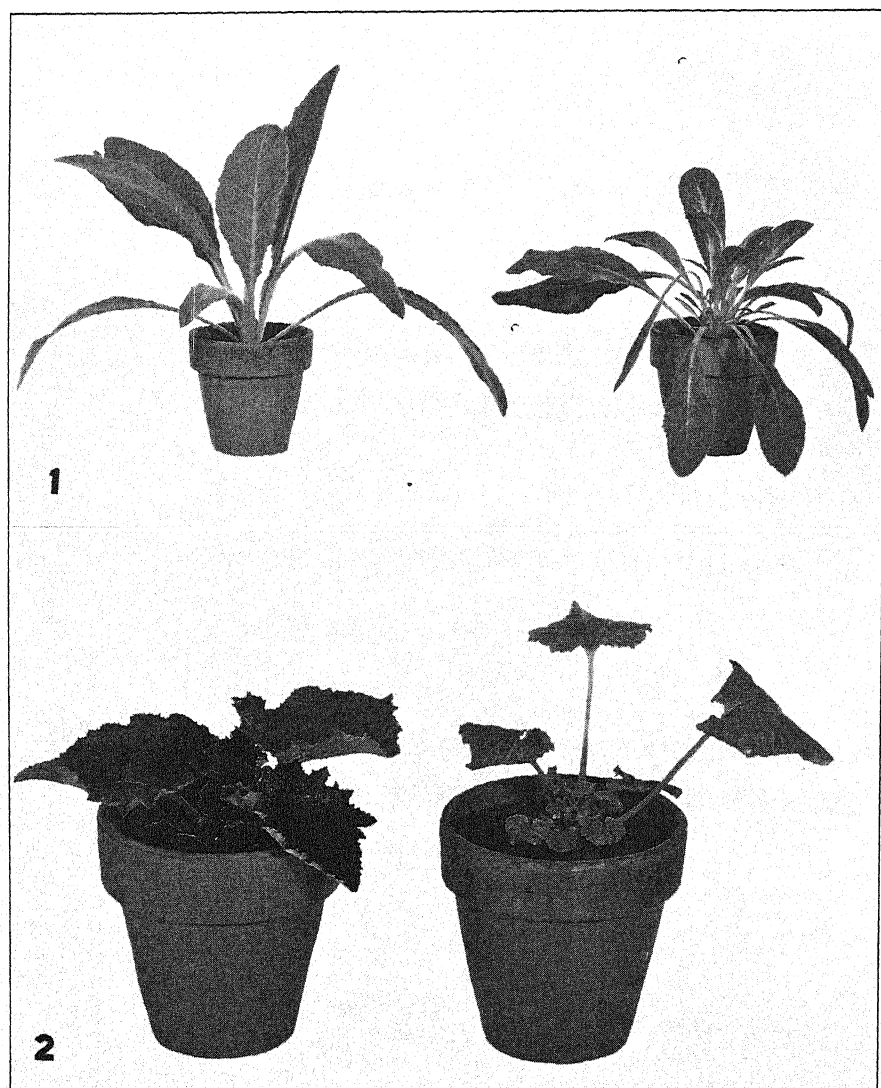
*Vaccaria segetalis* (Neck.) Gcke. Plants are dwarfed and much branched. They are moderately chlorotic.

TABLE I  
HOST RANGE OF ASTER YELLOWS

Name of plant	Family	Yellows transferred	
		From aster to new host	To aster from new host
<i>Humulus japonicus</i> Sieb. et Zucc.....	Moraceae	+	
<i>Monolepis chenopodioides</i> (Nutt.) Moq.....	Chenopodiaceae	+	
<i>Abronia umbellata</i> Lam. var. <i>grandiflora</i> ....	Nyctaginaceae	+	
<i>Tetragonia expansa</i> Murr.....	Aizoaceae	+	
<i>Dianthus alpinus</i> L.....	Caryophyllaceae	+	
<i>Herniaria glabra</i> L.....	"	+	
<i>Lychnis coronaria</i> Desr.....	"	+	
<i>Lychnis viscaria</i> L.....	"	+	
<i>Polycarpon tetraphyllum</i> L.....	"	+	
<i>Tunica saxifraga</i> (L.) Scop.....	"	+	
<i>Vaccaria segetalis</i> (Neck.) Gcke.....	"	+	
<i>Adonis aestivalis</i> L.....	Ranunculaceae	+	
<i>Papaver nudicaule</i> L.....	Papaveraceae	+	
<i>Cheiranthus Allionii</i> Hort.....	Cruciferae	+	
<i>Malcomia maritima</i> R. Br.....	"	+	
<i>Radicula sylvestris</i> (L.) Druce.....	"	+	
<i>Potentilla monspeliensis</i> L.....	Rosaceae	+	
<i>Limnanthes Douglasii</i> R. Br.....	Limnanthaceae	+	
<i>Datisca cannabina</i> L.....	Datisceae	+	
<i>Helianthemum chamaecistus</i> Mill.....	Cistaceae	+	
<i>Blumenbachia Hieronymi</i> Urb.....	Loasaceae	+	
<i>Cajophora lateritia</i> (Hook.) Kl.....	"	+	+
<i>Clarkia elegans</i> Doug.....	Onagraceae	+	+
<i>Ammi majus</i> L.....	Umbelliferae	+	
<i>Daucus carota</i> L.....	"	+	+
<i>Didiscus pusillus</i> F. et M.....	"	+	
<i>Levisticum paludapifolium</i> (Lam.) Aschs.....	"	+	
<i>Pastinaca sativa</i> L.....	"	+	+
<i>Armeria alpina</i> Willd.....	Plumbaginaceae	+	
<i>Limonium Suworowi</i> Kuntze.....	"	+	
<i>Anagallis limifolia</i> L.....	Primulaceae	+	
<i>Vinca rosea</i> L.....	Apocynaceae	+	+
<i>Gilia densiflora</i> Benth.....	Polemoniaceae	+	
<i>Gilia tricolor</i> Benth.....	"	+	
<i>Polemonium coeruleum</i> L.....	"	+	
<i>Phacelia campanularia</i> Gray.....	Hydrophyllaceae	+	
<i>Phacelia congesta</i> Hook.....	"	+	
<i>Phacelia viscida</i> Torr.....	"	+	
<i>Phacelia whillavia</i> Gray.....	"	+	
<i>Anchusa Barrelieri</i> Vilm.....	Boraginaceae	+	
<i>Anchusa capensis</i> Thumb.....	"	+	
<i>Dracocephalum Ruyschiana</i> L.....	Labiatae	+	
<i>Physostegia virginica</i> Benth.....	"	+	
<i>Browallia demissa</i> L.....	Solanaceae	+	
<i>Hyoscyamus niger</i> L.....	"	+	
<i>Nicotiana rustica</i> L.....	"	+	+
<i>Petunia hybrida</i> Vilm.....	"	+	
<i>Solanum lycopersicum</i> L.....	"	+	
<i>Alonsoa warscewiczii</i> Regel.....	Scrophylariaceae	+	
<i>Collinsia bicolor</i> Benth.....	"	+	
<i>Linaria cymbalaria</i> Mill.....	"	+	
<i>Linaria maroccana</i> Hook.....	"	+	
<i>Maurandia lophospermum</i> Bailey.....	"	+	
<i>Maurandia scandens</i> Pers.....	"	+	
<i>Verbascum hybridum</i> Hort.....	"	+	
<i>Veronica peregrina</i> L.....	"	+	
<i>Didymocarpus Horsfieldii</i> Schinz.....	Gesneriaceae	+	
<i>Thunbergia alata</i> Bojer.....	Acanthaceae	+	
<i>Plantago alpina</i> L.....	Plantaginaceae	+	
<i>Plantago fuscescens</i> Jord.....	"	+	
<i>Plantago psyllium</i> L.....	"	+	

TABLE I  
HOST RANGE OF ASTER YELLOWS (CONT.)

Names of plant	Family	Yellows transferred	
		From aster to new host	To aster from new host
<i>Centranthus calcitrapa</i> (L.) Dufr.....	Valerianaceae	+	
<i>Lobelia erinus</i> L. var. <i>compacta</i> .....	Campanulaceae	+	
<i>Acroclinium roseum</i> Hook.....	Compositae	+	
<i>Anthemis tinctoria</i> L.....	"	+	
<i>Arctotis grandis</i> Thumb.....	"	+	
<i>Cacalia hastata</i> L.....	"	+	
<i>Carthamus tinctorius</i> L.....	"	+	
<i>Chamae heterophylla</i> Cass.....	"	+	
<i>Chrysanthemum cinerariifolium</i> Bocc.....	"	+	+
<i>Cineraria hybrida</i> Hort.....	"	+	
<i>Cirsium oleraceum</i> (L.) Scop.....	"	+	
<i>Cladanthus arabicus</i> (L.) Cass.....	"	+	
<i>Coreopsis lanceolata</i> L.....	"	+	
<i>Cosmos bipinnatus</i> Cav.....	"	+	+
<i>Cousinia hystrix</i> C. A. Mey.....	"	+	
<i>Echinops dahuricus</i> Fisch.....	"	+	
<i>Emilia flammea</i> Cass.....	"	+	
<i>Erigeron glabellus</i> Nutt.....	"	+	
<i>Erigeron linifolius</i> Willd.....	"	+	
<i>Erigeron speciosus</i> (Lindl.) D. C.....	"	+	+
<i>Ethulia conyzoides</i> L.....	"	+	
<i>Eupatorium urticaefolium</i> Reich.....	"	+	
<i>Eupatorium perfoliatum</i> L.....	"	+	
<i>Felicia aethiopica</i> (Brum.) O. Hoffm. var. <i>glandulosa</i> .....	"	+	
<i>Felicia amelloides</i> Voss.....	"	+	
<i>Filago germanica</i> L.....	"	+	
<i>Flaveria repanda</i> Lag.....	"	+	
<i>Galinsoga parviflora</i> Cav.....	"	+	+
<i>Grindelia squarrosa</i> (Pursh.) Dun.....	"	+	
<i>Hedypnois cretica</i> (L.) Willd.....	"	+	
<i>Helenium autumnale</i> L.....	"	+	
<i>Helenium Biglovii</i> Gray.....	"	+	
<i>Helenium Hoopesii</i> Gray.....	"	+	
<i>Helenium nudiflorum</i> Nutt.....	"	+	
<i>Heliotropis laevis</i> Pers.....	"	+	
<i>Helipterum manglesii</i> Muell.....	"	+	
<i>Hieracium alpinum</i> L.....	"	+	
<i>Koelpinia linearis</i> Pall.....	"	+	
<i>Lagascea mollis</i> Cav.....	"	+	
<i>Leontodon autumnalis</i> L.....	"	+	+
<i>Leontopodium alpinum</i> Cass.....	"	+	
<i>Leptosyne Stillmani</i> Gray.....	"	+	
<i>Lindheimeria texana</i> A. Gr.....	"	+	
<i>Lonas inodora</i> (L.) Gaertn.....	"	+	
<i>Mulgedium alpinum</i> (L.) Less.....	"	+	
<i>Parthenium integrifolium</i> L.....	"	+	
<i>Petasites albus</i> (L.) Gaertn.....	"	+	
<i>Rudbeckia hirta</i> L.....	"	+	+
<i>Sanvitalia procumbens</i> Lam.....	"	+	
<i>Schkuhria abrotanoides</i> Roth.....	"	+	
<i>Scolymus hispanicus</i> L.....	"	+	
<i>Spilanthes acmella</i> (L.) Murr.....	"	+	
<i>Thelesperma hybridum</i> Voss.....	"	+	
<i>Tolpis barbata</i> Gaertn.....	"	+	
<i>Tragopogon floccosus</i> W. et K.....	"	+	
<i>Tridax trilobata</i> (Cav.) Hemsl.....	"	+	
<i>Ursinia arthemoides</i> (L.) Benth. et Hook.....	"	+	
<i>Zacyntha verrucosa</i> Gaertn.....	"	+	
<i>Zinnia multiflora</i> L.....	"	+	



FIGURES 1 and 2. Aster yellows disease on various species. FIG. 1. A healthy and a yellowed plant of *Verbascum hybridum* Hort. The yellowed plant has produced short secondary shoots. FIG. 2. A healthy and a yellowed plant of *Cineraria hybrida* Hort. The old leaves of the yellowed plant have long petioles; the young leaves are very small.

*Adonis aestivalis* L. Plants are dwarfed and chlorotic. They produce many secondary shoots after having the disease for a considerable period of time. An early stage of yellows on this host is shown in Figure 42.

*Papaver nudicaule* L. Plants are badly stunted and chlorotic.

*Cheiranthus Allionii* Hort. Plants are chlorotic and abnormally branched. The leaves are longer and narrower than the leaves of healthy plants as is shown in Figure 9.

*Malcomia maritima* R. Br. (Virginia stock). Plants show stunting but very little chlorosis. They produce many upright-growing secondary shoots. They bear virescent flowers out of the pistils of which long stems grow. These in turn bear secondary virescent flowers. A healthy and a yellowed plant are shown in Figure 31.

*Radicula sylvestris* (L.) Drūce. Plants are dwarfed and produce many slightly chlorotic secondary shoots.

*Potentilla monspeliensis* L. Plants are stunted and chlorotic. Clearing of veins of leaves is an early symptom. A healthy and a yellowed plant are shown in Figure 46.

*Limnanthes Douglasii* R. Br. Plants are chlorotic and produce many secondary shoots.

*Datisca cannabina* L. Plants are moderately chlorotic and produce leaves with greatly elongated petioles.

*Helianthemum chamaecistus* Mill. Plants are very chlorotic. They produce an abnormal number of secondary shoots.

*Blumenbachia Hieronymi* Urb. Plants are chlorotic.

*Cajophora lateritia* (Hook.) Kl. Plants produce chlorotic spindling secondary shoots and virescent flowers. The clearing of the veins of the leaves is an early symptom. A yellowed branch is shown in Figure 22.

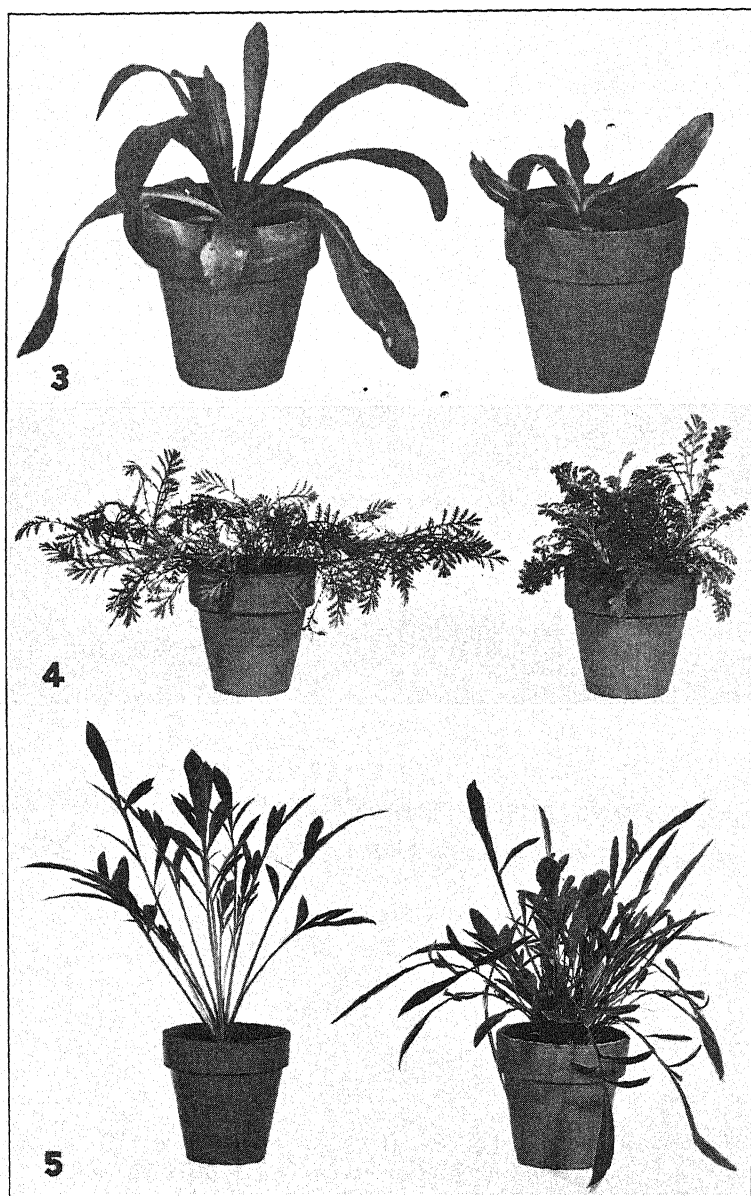
*Clarkia elegans* Doug. Plants are stunted and slightly chlorotic. The leaves at the tips of branches show chlorosis more plainly than the older leaves. The old leaves turn a reddish color. The branches grow in a more upright position than those of healthy plants as is shown in Figure 37. During the early stages of their growth it is not always easy to distinguish diseased from healthy plants. This difficulty disappears as soon as flowers are produced. The yellowed plants bear malformed virescent flowers.

*Ammi majus* L. Plants are chlorotic. They produce many secondary shoots and bear green flowers.

*Daucus carota* L. Plants produce chlorotic, upright-growing secondary shoots. The effect of the disease on a young plant of the cultivated carrot is shown in Figure 25. The leaves of the yellowed plant are in a more upright position than those of the healthy plant. The effect of yellows on a young plant of the wild carrot is shown in Figure 27.

*Didiscus pusillus* F. et M. Plants are chlorotic. Their branches grow in a somewhat more upright manner than the branches of healthy plants.





FIGURES 3-5. Aster yellows disease on various species. FIG. 3. A healthy and a yellowed plant of *Helenium Biglovii* Gray. The yellowed plant is badly dwarfed. FIG. 4. A healthy and a yellowed plant of *Anthemis tinctoria* L. The branches of the yellowed plant are more erect than those of the healthy one. FIG. 5. A healthy and a yellowed plant of *Coreopsis lanceolata* L. The yellowed plant has produced many short secondary shoots.

*Levisticum paludapifolium* (Lam.) Aschs. Plants are dwarfed but not chlorotic. They produce many secondary shoots as is shown in Figure 28. Diseased leaves are shorter and smaller than leaves of the same age on healthy plants.

*Pastinaca sativa* L. (cultivated parsnip). Plants are chlorotic and produce many secondary shoots. The leaves are smaller than those of healthy plants.

*Armeria alpina* Willd. Plants are chlorotic. They produce an abnormal number of secondary shoots.

*Limonium Suworowi* Kuntze. Plants are very chlorotic and dwarfed as is shown in Figure 41. Clearing of the veins in leaves is an early symptom. Old leaves become a reddish color; flowers are malformed and virescent.

*Anagallis linifolia* L. Plants are chlorotic and dwarfed. They produce many secondary shoots.

*Vinca rosea* L. Plants are slightly chlorotic. They produce upright-growing secondary shoots. The leaves show clearing of veins as an early symptom. Flowers are malformed and virescent. The plants are not much dwarfed. A healthy and a diseased plant are shown in Figure 43.

*Gilia densiflora* Benth. Plants are chlorotic and produce malformed virescent flowers. They are not severely dwarfed. A healthy and a diseased specimen are shown in Figure 44.

*Gilia tricolor* Benth. Plants are chlorotic and produce upright-growing secondary shoots as is shown in Figure 30. Flowers are green and malformed.

*Polemonium coeruleum* L. Plants are chlorotic.

*Phacelia campanularia* Gray. Plants are chlorotic and dwarfed as is shown in Figure 7. Malformed virescent flowers are produced.

*Phacelia congesta* Hook. Plants are dwarfed and chlorotic. They produce slender secondary shoots such as are shown in Figure 15. The flowers are malformed and virescent.

*Phacelia viscida* Torr. Plants are chlorotic and somewhat stunted as is shown in Figure 6. They produce upright-growing secondary shoots and green flowers.

*Phacelia whitlavia* Gray. Plants are dwarfed and chlorotic.

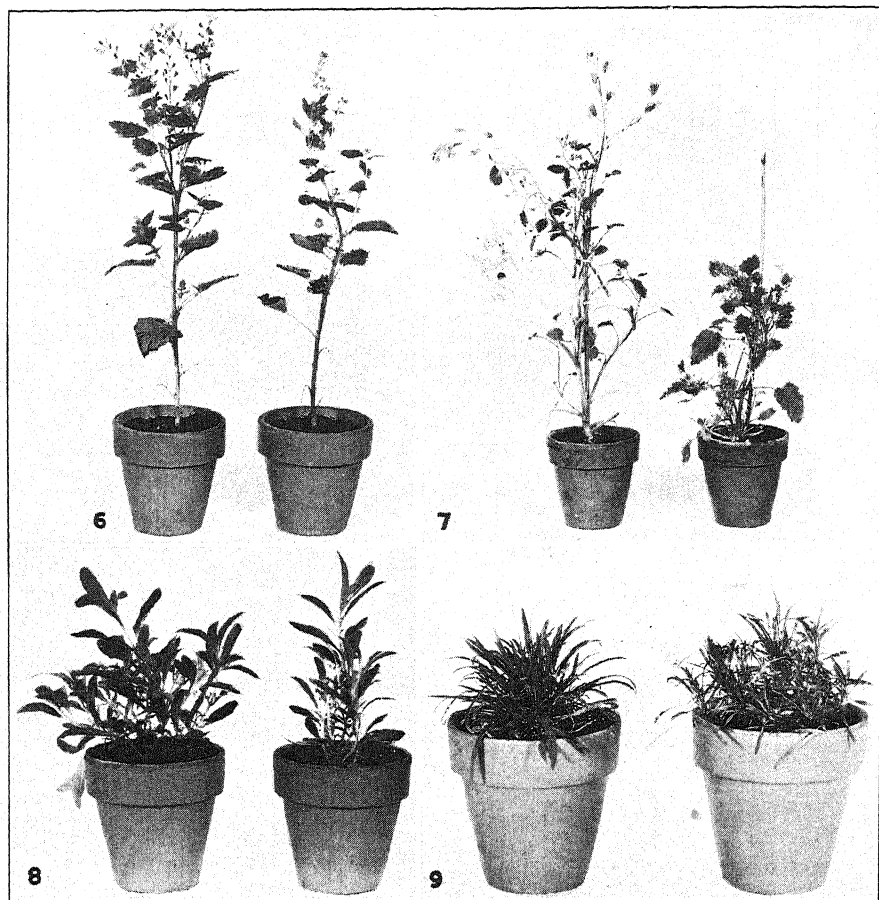
*Anchusa Barrelieri* Vilm. Plants are dwarfed and very chlorotic.

*Anchusa capensis* Thumb. Plants are dwarfed and chlorotic.

*Dracocephalum Ruyschiana* L. Plants are slightly chlorotic. They produce many secondary shoots.

*Physostegia virginica* Benth. Plants are chlorotic and produce many upright-growing secondary shoots.

*Browallia demissa* L. Plants are slightly chlorotic. They are greatly dwarfed as is shown in Figure 29.



FIGURES 6-9. Aster yellows disease on various species. FIG. 6. A healthy and a yellowed plant of *Phacelia viscida* Torr. The yellowed plant bears numerous virescent flowers. FIG. 7. A healthy and a yellowed plant of *Phacelia campanularia* Gray. The yellowed plant is stunted. It bears malformed green flowers. FIG. 8. A healthy and a yellowed plant of *Felicia amelloides* Voss. The yellowed plant shows a more upright habit of growth than the healthy one. FIG. 9. A healthy and a yellowed plant of *Cheiranthus Allionii* Hort. The yellowed plant bears short leaves and has branched more freely than the healthy plant.

*Hyoscyamus niger* L. Plants are slightly chlorotic.

*Nicotiana rustica* L. Plants are chlorotic and produce numerous secondary shoots as is shown in Figure 11.

*Petunia hybrida* Vilm. Plants are chlorotic and badly dwarfed. They bear malformed virescent flowers. There is much branching and rosetting of secondary shoots as is shown in Figure 38.

*Solanum lycopersicum* L. Plants are chlorotic, especially at the tips of the branches. They are stunted and produce numerous secondary shoots. Buds and short shoots are produced in the axils of leaflets. These symptoms are described in detail in a section on the transmission of yellows to the tomato.

*Alonsoa warscewiczii* Regel. Plants are chlorotic and produce an abnormal number of secondary shoots.

*Collinsia bicolor* Benth. Plants are chlorotic and produce numerous secondary shoots. Clearing of the veins in the leaves is an early symptom.

*Linearia cymbalaria* Mill. Plants are moderately chlorotic and much branched. They produce no flowers.

*Linaria maroccana* Hook. Plants are slightly chlorotic. They produce many upright-growing secondary shoots and bear virescent flowers.

*Maurandia lophospermum* Bailey. Plants are much dwarfed and are very chlorotic.

*Maurandia scandens* Pers. Plants are chlorotic. They produce numerous secondary shoots.

*Verbascum hybridum* Hort. Plants are chlorotic and dwarfed. They produce numerous upright-growing secondary shoots as is shown in Figure 1.

*Veronica peregrina* L. Plants are chlorotic. Many secondary shoots are produced.

*Didymocarpus Horsfieldii* Schinz. Plants are chlorotic and badly dwarfed. They produce an abnormal number of secondary shoots. The leaves are small.

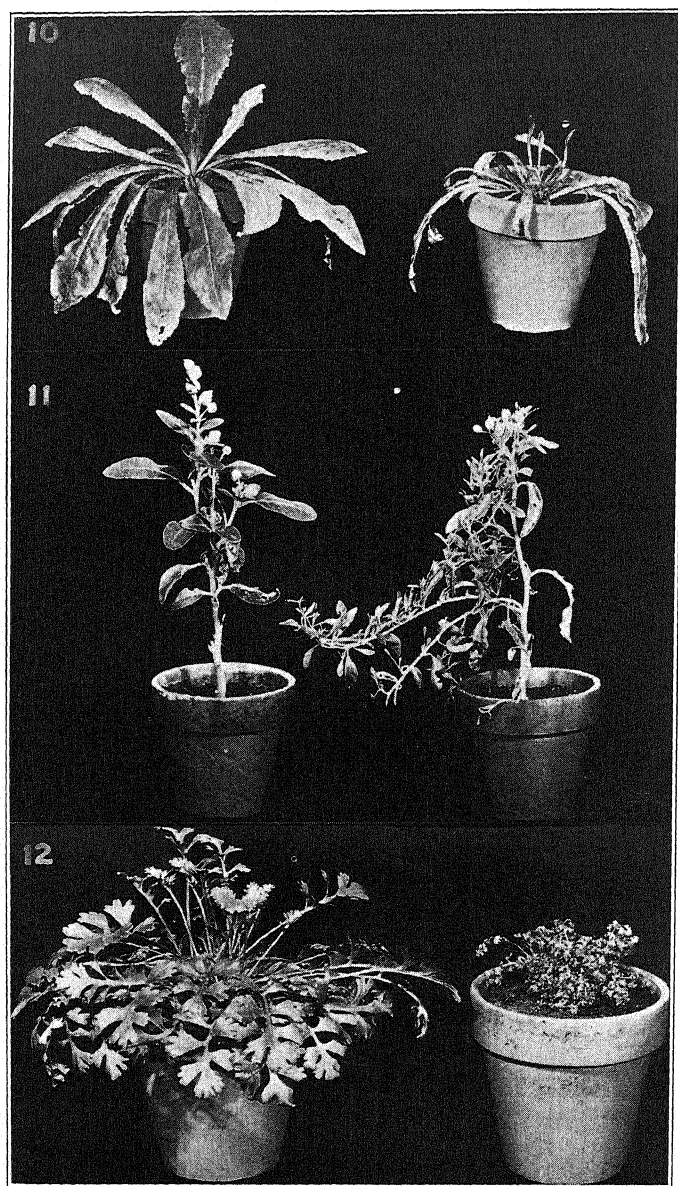
*Thunbergia alata* Bojer. Plants are chlorotic. The clearing of veins is an early symptom. Slender secondary branches and abnormal virescent flowers are produced. Plants are dwarfed as is shown in Figure 40.

*Plantago alpina* L. Plants are moderately chlorotic. Numerous secondary shoots are produced.

*Plantago fuscescens* Jord. Plants are very chlorotic.

*Plantago psyllium* L. Plants are chlorotic. They produce many slender secondary branches.

*Centranthus calcitrapa* (L.) Dufr. Plants are chlorotic and much dwarfed.



FIGURES 10-12. Aster yellows disease on various species. FIG. 10. A healthy and a yellowed plant of *Koelpinia linearis* Pall. The yellowed plant bears small leaves. FIG. 11. A healthy and a yellowed plant of *Nicotiana rustica* L. The yellowed plant has produced numerous slender branches. FIG. 12. A healthy and a yellowed plant of *Chrysanthemum cinerariifolium* Bocc. The yellowed plant is badly dwarfed.

*Lobelia erinus* L. var. *compacta*. Plants are chlorotic and produce many upright-growing secondary shoots. They bear malformed virescent flowers. A healthy and a yellowed plant are shown in Figure 32.

*Acroclinium roseum* Hook. Plants are dwarfed. The tips of the branches are chlorotic.

*Anthemis tinctoria* L. Plants are chlorotic. They produce numerous upright-growing shoots as is shown in Figure 4.

*Arcotis grandis* Thumb. Plants are chlorotic and much branched. They are greatly dwarfed as is shown in Figure 39.

*Cacalia hastata* L. Plants are very chlorotic. They produce malformed curled leaves as is shown in Figure 23.

*Carthamus tinctorius* L. Plants are chlorotic. They produce many secondary shoots. The flowers are malformed.

*Chorieis heterophylla* Cass. Plants are chlorotic and badly dwarfed as is shown in Figure 26.

*Chrysanthemum cinerariifolium* Bocc. (*Pyrethrum*). Plants are moderately chlorotic. They produce many secondary shoots and are dwarfed as is shown in Figure 12.

*Cineraria hybrida* Hort. Plants are chlorotic and dwarfed. They produce many short upright-growing secondary shoots and leaves with long petioles as is shown in Figure 2. Clearing of the veins is an early symptom.

*Cirsium oleraceum* (L.) Scop. Plants are chlorotic.

*Cladanthus arabiscus* (L.) Cass. Plants are dwarfed and chlorotic.

*Coreopsis lanceolata* L. Plants are not chlorotic but produce numerous secondary shoots as is shown in Figure 5.

*Cosmos bipinnatus* Cav. Plants are chlorotic. They produce upright-growing secondary shoots and green flowers. A healthy and a yellowed branch are shown in Figure 17.

*Cousinia hystrix* C. A. Mey. Plants are chlorotic and show a more upright habit of growth than is normal for this species.

*Echinops dahuricus* Fisch. Plants are dwarfed and chlorotic.

*Emilia flammea* Cass. Plants are very chlorotic.

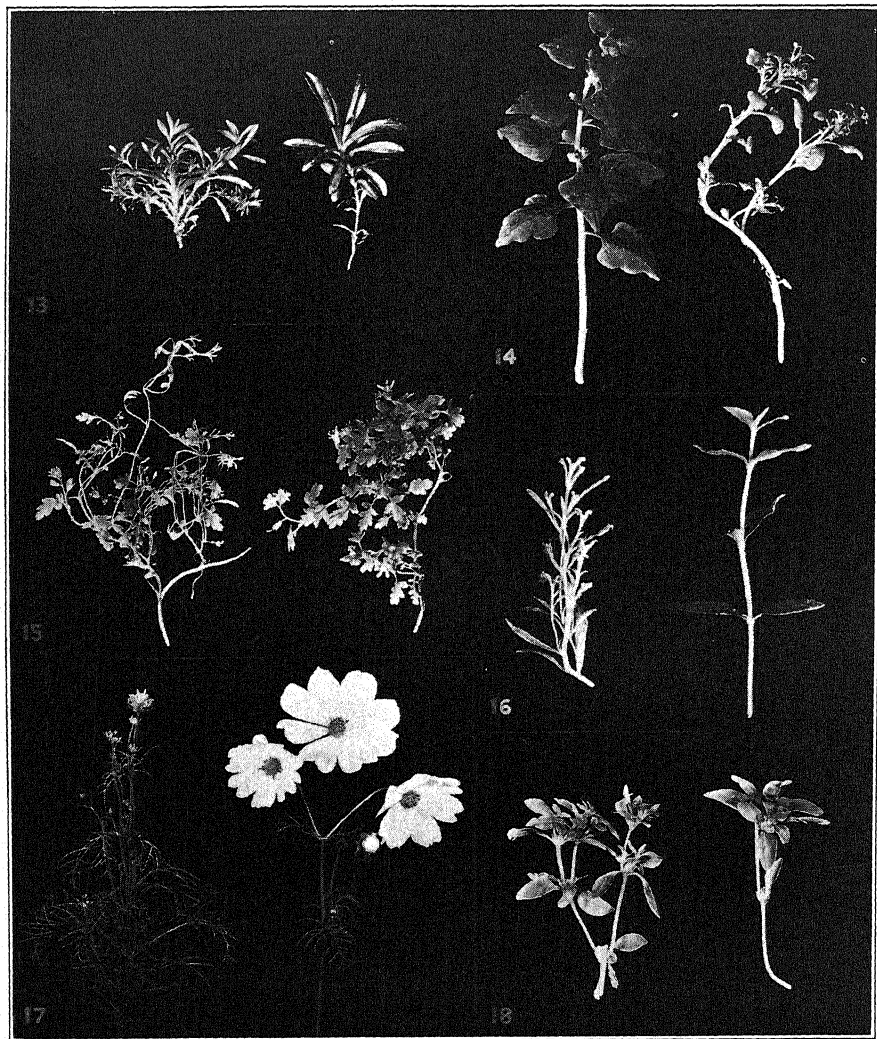
*Erigeron glabellus* Nutt. Plants are chlorotic. They produce many secondary shoots.

*Erigeron linifolius* Willd. Plants show marked chlorosis. They produce many upright-growing secondary shoots and are badly stunted as is shown in Figure 19.

*Erigeron speciosus* (Lindl.) D. C. Plants are very chlorotic. They produce many secondary shoots and bear green flowers.

*Ethulia conyzoides* L. Plants are chlorotic and produce slender secondary shoots. A healthy and a yellowed branch are shown in Figure 16.

*Eupatorium urticaefolium* Reich. Plants are chlorotic. They produce numerous upright-growing secondary shoots.



FIGURES 13-18. Aster yellows disease on various species. FIG. 13. A healthy and a yellowed branch of *Dianthus alpinus* L. The diseased branch bears small leaves and long secondary shoots. FIG. 14. A healthy and a diseased branch of *Tetragonia expansa* Murr. The diseased branch bears small leaves and slender secondary shoots. FIG. 15. A healthy and a yellowed branch of *Phacelia congesta* Hook. The yellowed branch bears slender secondary shoots and green flowers. FIG. 16. A healthy and a yellowed branch of *Ethulia conyzoides* L. The yellowed branch has produced numerous upright-growing secondary shoots. FIG. 17. A healthy and a yellowed branch of *Cosmos bipinnatus* Cav. The yellowed branch bears imperfect flowers. FIG. 18. A healthy and a yellowed branch of *Felicia aethiopica* (Brum.) O. Hoffm. var. *glandulosa*. The yellowed branch bears short leafy shoots instead of flowers.

*Eupatorium perfoliatum* L. Plants are chlorotic.

*Felicia aethiopica* (Brum.) O. Hoffm. var. *glandulosa*. Plants show marked chlorosis and an upright habit of growth. They do not produce flowers. A healthy and a yellowed branch are shown in Figure 18.

*Felicia amelloides* Voss. Plants are chlorotic. They produce slender secondary shoots as is shown in Figure 8.

*Filago germanica* L. Plants are dwarfed and chlorotic. They produce numerous small secondary shoots as is shown in Figure 21.

*Flaveria repanda* Lag. Plants are chlorotic.

*Galinsoga parviflora* Cav. Plants are chlorotic. They produce slender secondary shoots and green flowers.

*Grindelia squarrosa* (Pursh.) Dun. Plants are dwarfed and chlorotic.

*Hedypnois cretica* (L.) Willd. Plants are moderately chlorotic. They produce numerous upright-growing secondary shoots.

*Helenium autumnale* L. Plants are chlorotic. Numerous secondary shoots are produced. The leaves have longer petioles than the leaves of healthy specimens. Plants bear virescent flowers.

*Helenium Biglovii* Gray. Plants are chlorotic and dwarfed as is shown in Figure 3.

*Helenium Hoopesii* Gray. Young leaves are chlorotic. Old leaves are a light reddish color.

*Helenium nudiflorum* Nutt. Plants are chlorotic and dwarfed. The leaves show clearing of veins as an early symptom. Numerous upright-growing shoots and virescent flowers are produced.

*Heliopsis laevis* Pers. Plants are chlorotic and dwarfed.

*Helipterum manglesii* Muell. Plants are chlorotic. They are dwarfed and produce many secondary shoots.

*Hieracium alpinum* L. Plants are chlorotic.

*Koelerpinia linearis* Pall. Plants are chlorotic and dwarfed as is shown in Figure 10.

*Lagascea mollis* Cav. Plants are chlorotic and much dwarfed. Many upright shoots are produced as is shown in Figure 24.

*Leontodon autumnalis* L. Plants are chlorotic and much dwarfed as is shown in Figure 34. Malformed virescent flowers are produced.

*Leontopodium alpinum* Cass. Plants are chlorotic and show an upright habit of growth.

*Leptosyne Stillmani* Gray. Plants are chlorotic and dwarfed as is shown in Figure 36.

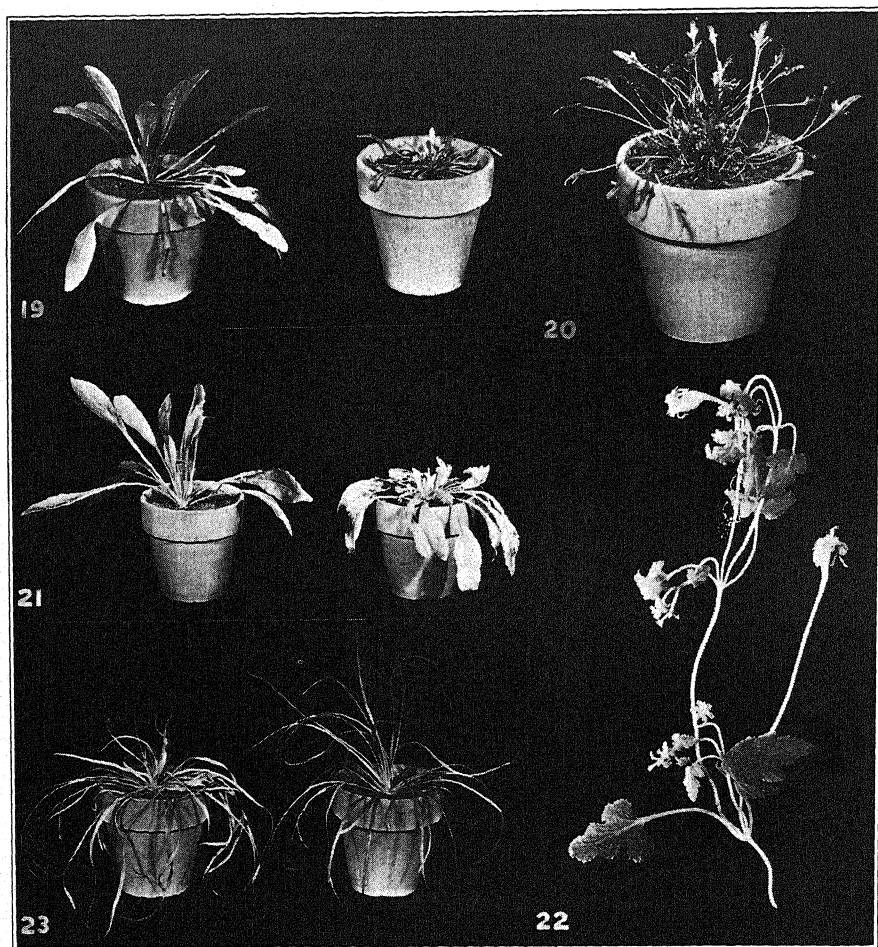
*Lindheimeria texana* A. Gr. Plants are slightly chlorotic. Leafy malformed virescent flowers are produced.

*Lonas inodora* (L.) Gaertn. Plants are chlorotic and dwarfed.

*Mulgedium alpinum* (L.) Less. Plants are chlorotic.

*Parthenium integrifolium* L. Plants are very chlorotic and dwarfed.





FIGURES 19-23. Aster yellows disease on various species. FIG. 19. A healthy and a yellowed plant of *Erigeron linifolius* Willd. The yellowed plant is badly stunted. FIG. 20. A yellowed plant of *Monolepis chenopodioides* (Nutt.) Moq. The plant has produced a tuft of short branches. FIG. 21. A healthy and a yellowed plant of *Filago germanica* L. The yellowed plant has produced short secondary shoots. FIG. 22. A yellowed branch of *Cajo-phora lateritia* (Hook.) Kl., showing slender side shoots and malformed virescent flowers. FIG. 23. A healthy and a yellowed plant of *Cacalia hastata* L. The yellowed plant is stunted and chlorotic.

*Petasites albus* (L.) Gaertn. Plants are chlorotic. They produce slender upright-growing shoots.

*Rudbeckia hirta* L. Plants are chlorotic and produce malformed virescent flowers.

*Sanvitalia procumbens* Lam. Plants are very chlorotic.

*Schkuhria abrotanoides* Roth. Plants are dwarfed and chlorotic.

*Scolymus hispanicus* L. Plants are chlorotic.

*Spilanthes acmella* (L.) Murr. Plants are dwarfed and chlorotic.

*Thelesperma hybridum* Voss. Plants are chlorotic. They produce many secondary shoots.

*Tolpis barbata* Gaertn. Plants are chlorotic and dwarfed as is shown in Figure 33. Clearing of the veins of the leaves is an early symptom. Virescent flowers are produced.

*Tragopogon floccosus* W. et K. Plants are chlorotic. Many secondary shoots are produced.

*Tridax trilobata* (Cav.) Hemsl. Plants are chlorotic and bear virescent flowers. The leaves show clearing of veins as an early symptom.

*Ursinia anthemoides* (L.) Benth. et Hook. Plants are chlorotic and dwarfed.

*Zacyntha verrucosa* Gaertn. Plants are chlorotic and produce many secondary shoots.

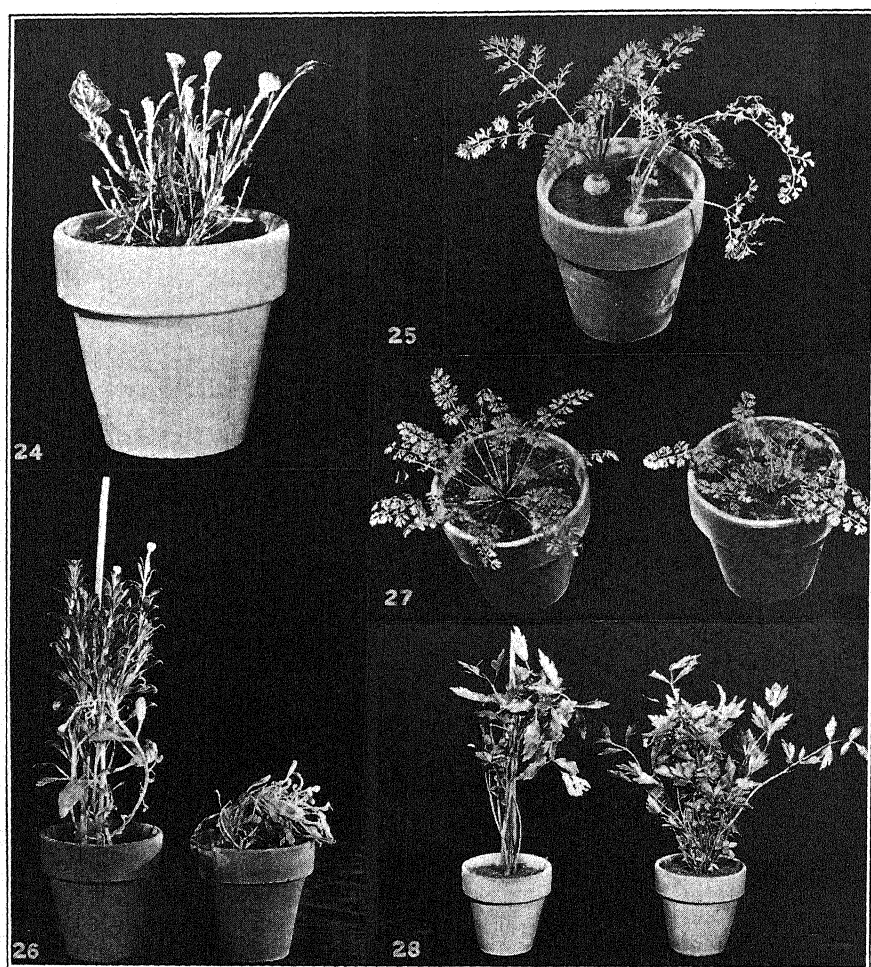
*Zinnia multiflora* L. Plants are chlorotic. They produce numerous upright-growing secondary shoots.

So far as the writer is aware there is no record of the natural occurrence of yellows on most of the plants here listed as hosts of aster yellows. A yellows disease showing symptoms indistinguishable from those of aster yellows and believed to be identical with this disease was, however, observed occurring naturally on several of the species listed.

Yellowed plants of *Potentilla monspeliensis* were received from Dr. H. H. Whetzel. He found them growing as weeds in Ithaca, New York, and near Camden, New York.

Yellowed specimens of *Clarkia elegans* were received from a grower who reported a loss of about one-third of his plants. Virus-free leafhoppers were allowed to feed on some of these plants and the disease was transmitted to asters. It proved to be aster yellows.

A yellows disease occurs commonly but not very abundantly on the weed, *Daucus carota*. It also occurs naturally on the cultivated carrot which belongs to the same species. It was transmitted from the carrot to the aster and shown to be identical with aster yellows. Aster yellows, transmitted experimentally to both the wild and the cultivated form of the species, causes symptoms identical with those shown by plants naturally infected. Severin (11) has reported the transmission of a yellows disease occurring in California from the aster to the cultivated carrot and from the cultivated



FIGURES 24-28. Aster yellows disease on various species. FIG. 24. A yellowed plant of *Lagascea mollis* Cav. The plant has produced a large tuft of short branches. FIG. 25. A healthy and a yellowed plant of the cultivated carrot, *Daucus carota* L. The yellowed plant shows an early stage of the disease. FIG. 26. A healthy and a yellowed plant of *Charieis heterophylla* Cass. The yellowed plant has produced many short secondary shoots. FIG. 27. A healthy and a yellowed plant of the wild carrot, *Daucus carota* L. The yellowed plant bears a tuft of secondary shoots. FIG. 28. A healthy and a yellowed plant of *Levisticum paludapifolium* (Lam.) Aschs. The yellowed plant bears many small leaves.

carrot to the aster. There is some question as to whether this disease is identical with aster yellows since it goes readily to celery (*Apium graveolens* L.) and to *Zinia elegans*, plants that are highly resistant if not immune to aster yellows.

Yellowed plants of the parsnip (*Pastinaca sativa*) have frequently been observed in gardens in the vicinity of Yonkers, New York. This yellows was transmitted to the aster. It caused symptoms identical with those of aster yellows. When aster yellows was transmitted to pot-grown parsnips it caused symptoms identical with those shown by plants naturally infected.

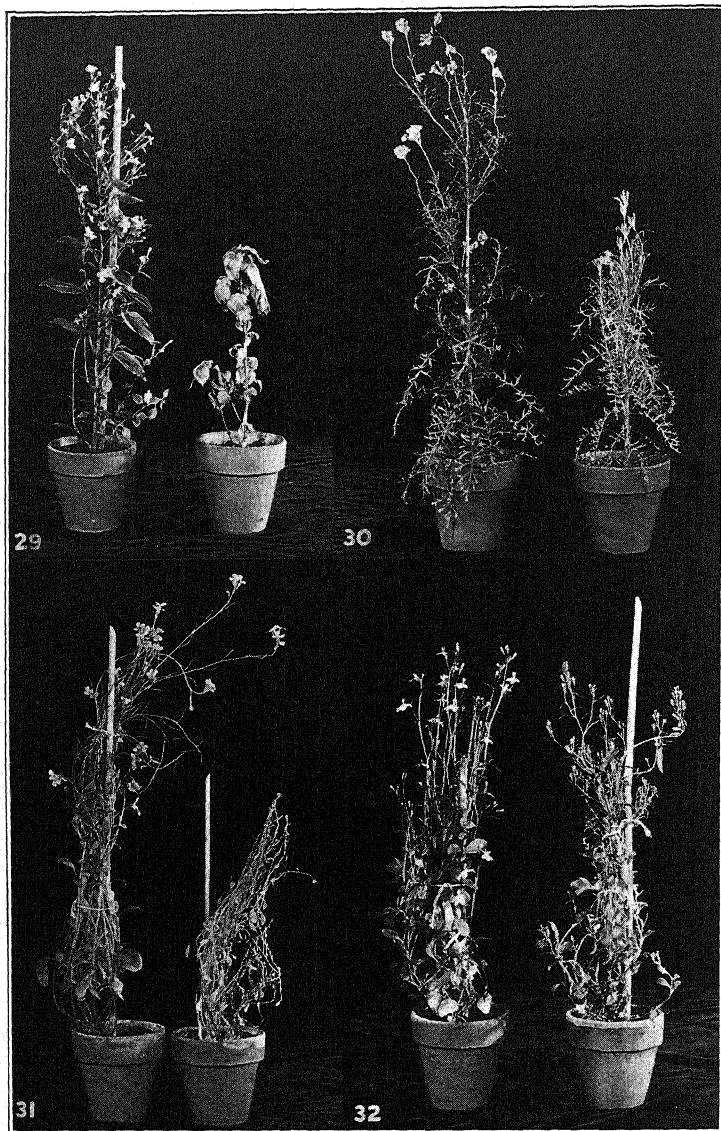
The writer has not found yellows occurring naturally on *Vinca rosea*. Reports from others indicate that a disease which is probably aster yellows is common on this plant when it is grown in flower gardens.

A yellows disease was received from a commercial grower of *Chrysanthemum cinerariifolium*, a species from which pyrethrum is obtained. When aster yellows was transmitted to healthy seedlings it caused symptoms identical with those shown by the field-grown plants.

A yellowed plant of *Rudbeckia hirta* was received from Dr. A. F. Blakeslee who found it growing wild. When transmitted to the aster the disease caused symptoms identical with those of aster yellows. Aster yellows was transmitted to healthy *Rudbeckia* plants. It caused symptoms similar to those observed on the wild plant.

In 1927 yellowed plants of *Leontodon autumnale* (fall dandelion) were received from Dr. Donald Folsom who called attention to a statement in Gray's New Manual of Botany to the effect that a frequent teratological or pathological form of this species has greenish yellow beads without ligules. The disease on this plant was first described by Morse (7) who found it occurring abundantly on lawns of the University of Maine. The plant is not a common weed in the vicinity of Yonkers, New York. Nevertheless, a few yellowed specimens were found in the summer of 1929. When yellows was transmitted from one of these specimens to aster plants it caused symptoms identical with those of aster yellows. Likewise, when aster yellows was experimentally transmitted to seedling *Leontodon* plants it caused symptoms similar to those shown by the plants found growing as weeds in Yonkers and also similar to the symptoms shown by the plants received from Dr. Folsom. The disease described by Morse and reported as common on the fall dandelion in Maine is doubtless aster yellows.

*Nicotiana rustica*, *Thunbergia alata*, *Cineraria hybrida*, *Coreopsis lanceolata*, and *Cosmos bipinnatus* have been observed to take yellows naturally when grown in a garden at Yonkers, New York. *Erigeron speciosus*, *Galinsoga parviflora*, *Helenium autumnale*, and *Helenium nudiflorum* are common weeds which are occasionally attacked by a yellows disease. Aster yellows was transmitted to plants of each of these species. It caused symptoms identical with those shown by plants in nature.



FIGURES 29-32. Aster yellows disease on various species. FIG. 29. A healthy and a yellowed plant of *Browallia demissa* L. The yellowed plant is badly dwarfed. FIG. 30. A healthy and a yellowed plant of *Gilia tricolor* Benth. The yellowed plant is stunted and bears green flowers. FIG. 31. A healthy and a yellowed plant of *Malcomia maritima* R. Br. The yellowed plant bears small virescent flowers and is beginning to die. FIG. 32. A healthy and a yellowed plant of *Lobelia erinus* L. var. *compacta*. The yellowed plant bears many virescent flowers.

A yellows disease was found in considerable abundance on *Eupatorium serotinum* Michx. in the Middle West. This plant is the common white weed or boneset of that region. It occurs in large numbers in pastures in Missouri, Illinois, Indiana, and the western half of Ohio. It has not been collected by the writer east of Ohio. The disease on this species causes marked chlorosis and stunting. It does not, however, cause the production of large numbers of secondary shoots. Although diseased plants show a more upright habit of growth than do healthy ones, this symptom is not so marked as it is for aster yellows on most host plants. The disease also causes a somewhat whiter type of chlorosis than is typical for aster yellows. Since, however, aster yellows was experimentally transmitted to two other species of *Eupatorium* it was at first thought that the disease occurring so abundantly on *Eupatorium serotinum* in the Middle West was probably aster yellows.

In an attempt to obtain evidence in support of this view many healthy young seedlings were grown in a greenhouse and exposed for varying periods of time to virus-bearing colonies of *Cicadula sexnotata*. In no case did they take the aster yellows disease. Early in May some 25 healthy seedlings were transplanted to a garden containing a large plot of asters. The aster plants became badly diseased with yellows as the season advanced. All of the *Eupatorium* plants remained healthy. Some of the potted plants were kept in a greenhouse for several years and were repeatedly exposed to leafhoppers bearing the aster yellows virus. They did not become diseased. Since the yellows occurring on this plant in the Middle West is so abundant as to indicate a high degree of susceptibility and since it was not possible to transmit aster yellows to it experimentally, the conclusion is reached that this *Eupatorium* yellows is probably distinct from aster yellows.

#### RESISTANT OR IMMUNE PLANTS

Plants belonging to a large number of different species remained healthy after exposure to virus-bearing leafhoppers under conditions favorable for transmission. In many instances they were exposed repeatedly. These plants are either immune or highly resistant. The many species to which they belong need not be listed here. Experiments dealing with a few that are of special interest are described below.

#### TOMATO (*SOLANUM LYCOPERSICUM* L.)

Several attempts were made to transmit aster yellows to the tomato during the summer of 1925. *Cicadula sexnotata* does not breed and flourish on this plant. It can live on tomatoes for a number of days if forced to feed on them. In no case did plants exposed to virus-carrying insects develop symptoms of aster yellows. It was, therefore, concluded that the tomato is highly resistant if not immune to the disease.

During the summer of 1928 Prof. J. B. S. Norton sent the writer some





FIGURES 33-36. Aster yellows disease on various species. FIG. 33. A healthy and a yellowed plant of *Tolpis barbata* Gaertn. The yellowed plant bears green flowers on numerous slender branches. FIG. 34. A healthy and a yellowed plant of *Leontodon autumnalis* L. The yellowed plant is badly dwarfed. FIG. 35. A healthy and a yellowed plant of *Lychnis viscaria* L. The yellowed plant is stunted and bears abnormal flowers. FIG. 36. A healthy and a yellowed plant of *Leptosyne Stillmani* Gray. The diseased plant is stunted. It has produced slender secondary shoots.

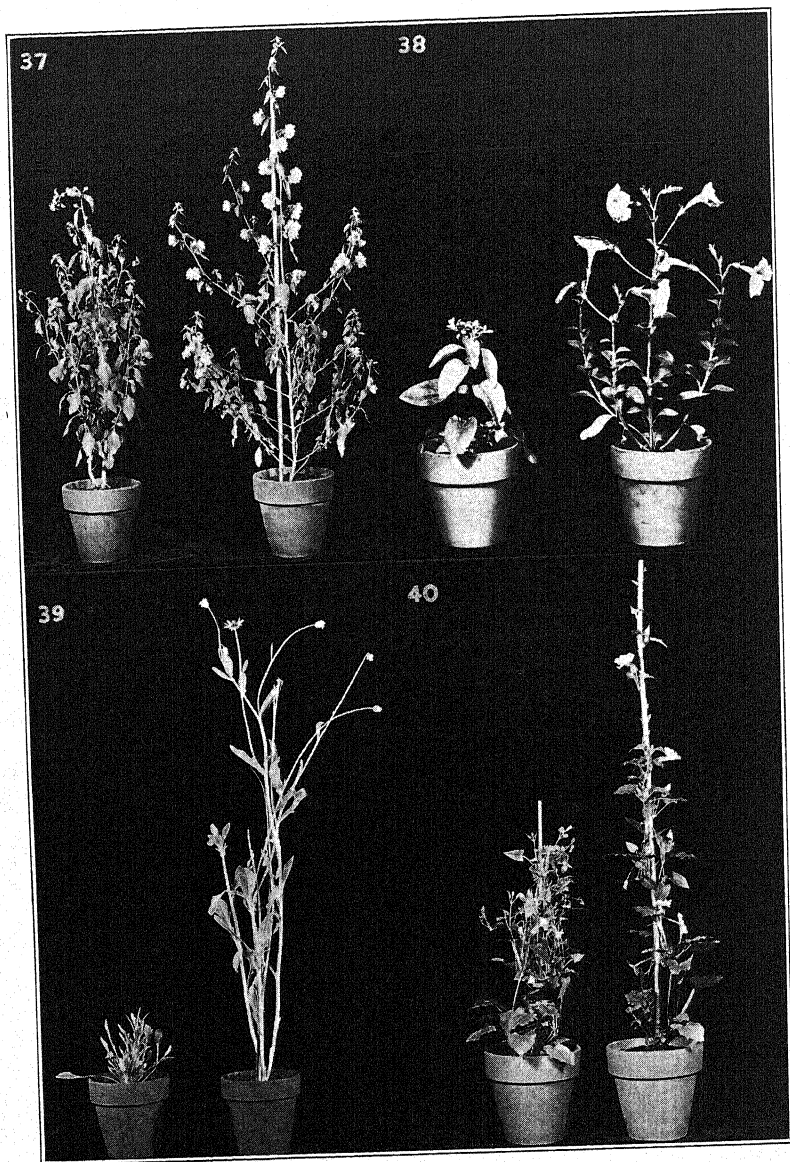
tomato plants from Salisbury, Maryland, which he suggested might have the aster yellows disease. The plants were slightly chlorotic. They showed a bushy type of growth and were considerably dwarfed. Norton stated that usually not more than one in every 500 to 1000 plants takes the disease. In one place, however, near the edge of a field he found as much as four per cent of the plants affected.

Since a number of solanaceous plants take aster yellows it was thought that the disease might be transmitted to tomatoes by grafting from diseased specimens of some closely related plant although it is not readily transmitted by the leafhopper. *Nicotiana rustica* was chosen for this purpose. A brief report of experiments with this plant has already been published (6). Yellowed buds were transplanted to 12 healthy tomato plants of the Bonnie Best variety. In five of the plants the buds lived and grew. The abundant growth produced by one of the *N. rustica* buds is shown in Figure 45. All of the five plants in which *N. rustica* buds lived produced the bushy type of growth shown in Figure 45. The branches developed chlorotic tips. There was a marked downward curling of the larger leaves. The plants grew very little after taking the disease. None of those plants in which the yellowed buds failed to grow showed any symptoms of disease. Plants to which healthy buds of *N. rustica* were transplanted also remained healthy.

Yellows symptoms in plants to which yellowed buds have been transplanted might result from actual transmission of the disease or from the passage of some toxic substance from the transplanted tissues into the affected plant. The latter possibility seemed to be a somewhat likely one, since the *N. rustica* buds grew into rather large branches. In the hope of obtaining evidence on this point the *N. rustica* tissues were cut from one of the plants. The plant so treated did not recover, but continued to show the symptoms of yellows for several months and until it died. It is, therefore, concluded that the disease was actually transmitted to the tomato.

The yellowed tomato plants showed one symptom which has not been observed in any of the other species to which yellows was transmitted. The disease stimulated the production of leafy buds and branches in the axils of many of the leaflets of yellowed plants. The abnormal production of secondary shoots is one of the most common symptoms of aster yellows. The disease stimulates growth in buds that would remain dormant in healthy plants. This results in a witches' broom type of growth. In the tomato the disease not only causes the abnormal production of secondary shoots as is shown by the branch pictured in Figure 49, but it stimulates the development of leafy buds and shoots in the axils of leaflets as well. A diseased leaf bearing several leafy shoots is shown in Figure 50. One of the plants, to which the disease was transmitted, produced shoots in the axils of 11 different leaflets. Such shoots were produced by all of the five yel-





FIGURES 37-40. Aster yellows disease on various species. FIG. 37. A healthy and a yellowed plant of *Clarkia elegans* Doug. The diseased plant is stunted. Its branches are somewhat more erect than the branches of the healthy plant. FIG. 38. A healthy and a yellowed plant of *Petunia hybrida* Vilm. The yellowed plant grew very little after taking the disease. FIG. 39. A healthy and a yellowed plant of *Arctotis grandis* Thumb. The yellowed plant is very badly dwarfed. FIG. 40. A healthy and a yellowed plant of *Thunbergia alata* Bojer. The yellowed plant bears malformed green flowers. Many slender secondary shoots have been produced.

lowed plants. They were not produced by the leaves of the healthy check plants of the same age and variety that were held under the same growing conditions as were the yellowed plants.

The normal tomato plant shows a tendency to produce buds in the axils of its leaflets. Buds and shoots are reported to occur occasionally on the leaves of healthy plants. Duchartre (2) described this phenomenon many years ago. It is interesting that aster yellows which quite generally stimulates growth in dormant buds should cause shoots to grow from tomato leaves.

It is believed that the disease observed by Prof. Norton in Maryland was aster yellows. Under very favorable conditions *Cicadula sexnotata* may transmit yellows to the tomato. The incidence of the disease in Maryland is such as to make this explanation seem probable. Further efforts will be made to transmit aster yellows to the tomato by means of *Cicadula sexnotata*.

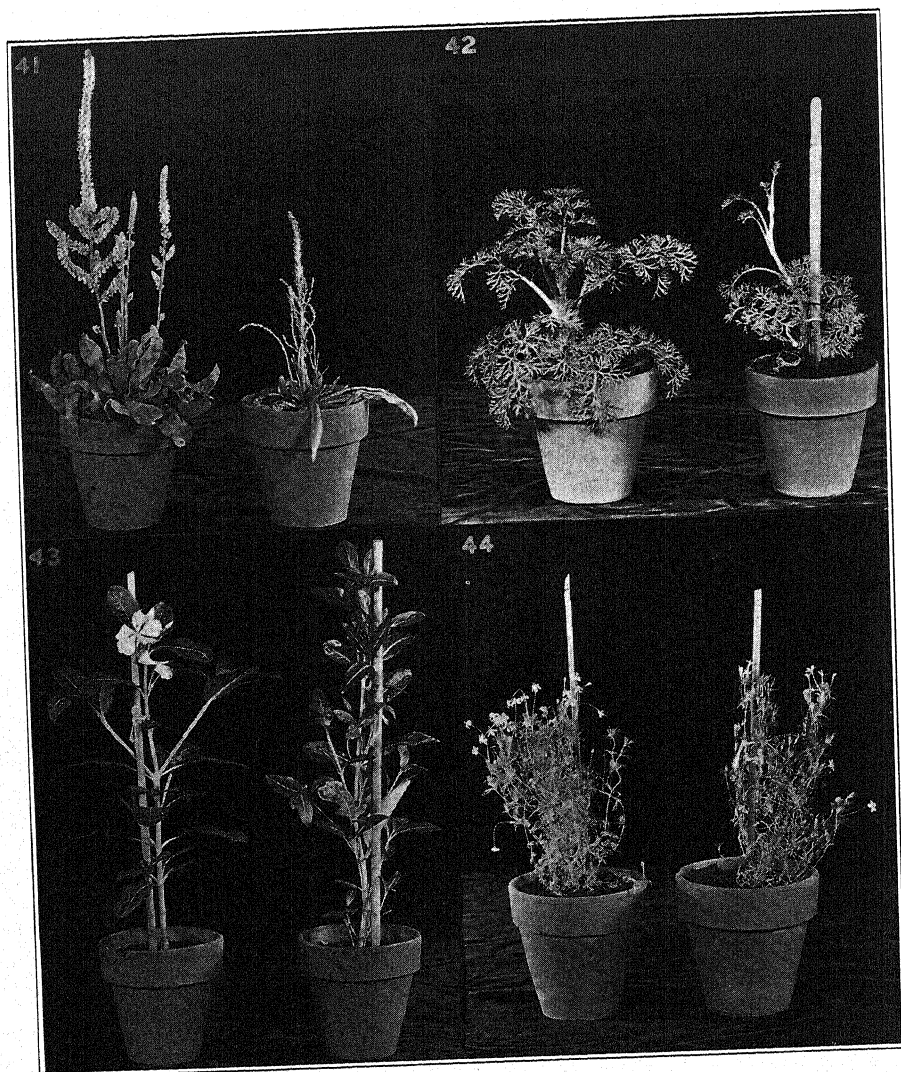
#### JIMSON WEED (*DATURA STRAMONIUM* L.)

Yellowed buds of *N. rustica* were transplanted to healthy seedlings of *Datura stramonium*. They grew almost as well on this plant as they did on the tomato. Yellows was not transmitted. It is, therefore, concluded that this species is immune under the conditions of the experiment.

#### POTATO (*SOLANUM TUBEROSUM* L.)

Numerous attempts have been made to transmit aster yellows to the potato by means of the insect vector. The varieties used include Irish Cobbler, Green Mountain, Bliss Triumph, and Spaulding Rose. The leafhopper was found to prefer Spaulding Rose to the other varieties. In one experiment a colony was kept for 16 days on plants of this variety without the loss of an unduly large number of insects. The Green Mountain variety proved to be an unsuitable host plant. A considerable number of insects always died when colonies were compelled to feed on it for more than five days. In no case was yellows transmitted to the potato.

The witches' broom disease of the potato causes symptoms similar to those that are typical for aster yellows on many of its host plants. It causes the production of large numbers of slender secondary shoots. Plants are stunted and more or less chlorotic. Because of the resemblance of this disease to aster yellows an attempt was made to transmit it to the aster by means of *Cicadula sexnotata* (5). Virus-free colonies were forced to feed for three days on potato plants having the witches' broom disease. They were then confined on healthy aster plants for a period of five weeks. The aster plants remained healthy. The experiments indicate that aster yellows is distinct from witches' broom of potato and that the potato varieties tested are either immune or highly resistant to the aster yellows disease. No attempt was made to transmit aster yellows to the potato by means of grafting.



FIGURES 41-44. Aster yellows disease on various species. FIG. 41. A healthy and a yellowed plant of *Limonium Suworowi* Kuntze. The yellowed plant bears small virescent flowers. FIG. 42. A healthy and a yellowed plant of *Adonis aestivalis* L. The leaves of the yellowed plant are smaller and shorter than those of the healthy plant. FIG. 43. A healthy and a yellowed plant of *Vinca rosea* L. The branches of the yellowed plant are somewhat more erect than those of the healthy plant. FIG. 44. A healthy and a yellowed plant of *Gilia densiflora* Benth. The yellowed plant bears many virescent flowers.

TOBACCO (*NICOTIANA TABACUM* L.)

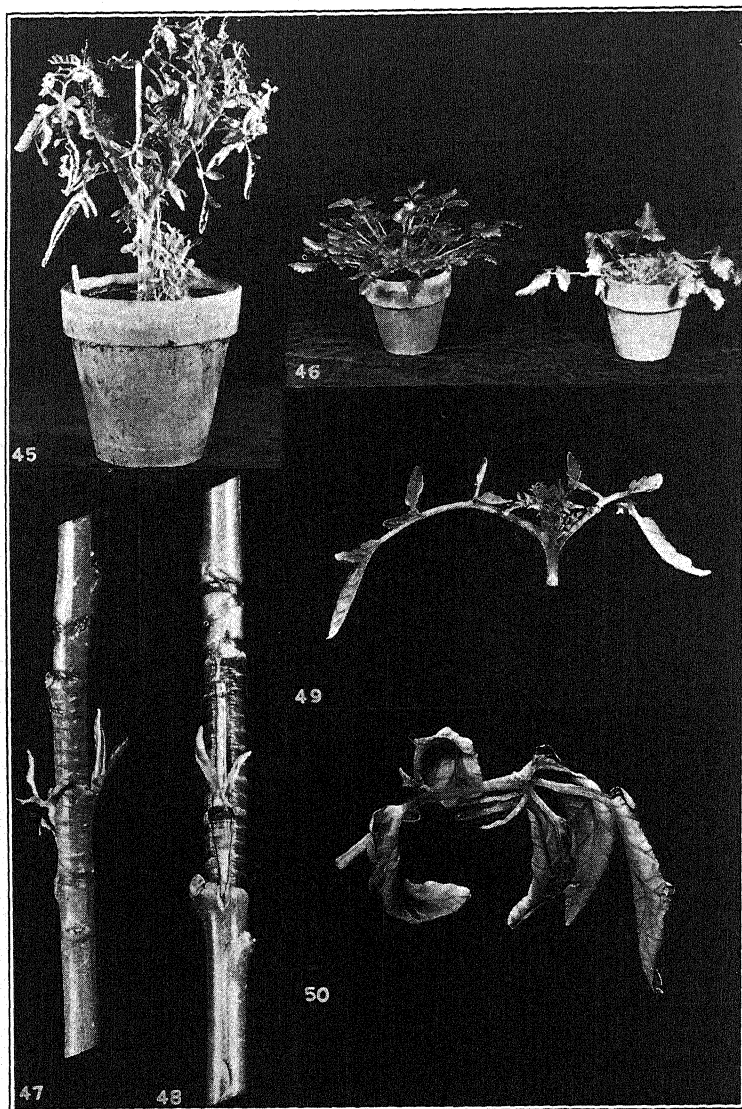
Tobacco plants were repeatedly exposed to virus-bearing colonies of *Cicadula sexnotata*. In no case did they develop symptoms of disease. Yellowing is readily transmitted to *N. rustica* as was reported in Table I. It was thought that the disease might be taken to tobacco by grafting diseased *N. rustica* tissues on healthy tobacco plants. Yellowed buds of *N. rustica* were accordingly transplanted to six healthy tobacco plants. The yellowed buds lived and grew in four of the plants. They remained alive for several months and produced a bushy type of growth similar to that produced on the tomato. The disease was not transmitted. It is, therefore, concluded that the tobacco is immune to aster yellows.

Since *N. rustica* is one of the plants to which the tobacco mosaic disease is readily transmitted mechanically by means of juice, it was thought that aster yellows might also be transmitted to this species in the same manner. Freshly expressed juice from yellowed *N. rustica* plants was pricked into 24 healthy *N. rustica* seedlings. Several hundred pin pricks were made in the leaves of each plant. Similar samples of juice were rubbed, according to the method described by Holmes (3) for the transmission of tobacco mosaic, over the upper surface of each of three mature young leaves on each of 24 other healthy *N. rustica* seedlings. Bits of tissue from yellowed *N. rustica* leaves were inserted into wounds in the stems of 24 additional healthy *N. rustica* seedlings. Twenty-four uninoculated seedlings served as checks. All plants were held under observation for a period of four weeks after inoculation. The experiment was then ended. The plants remained healthy. Aster yellows is not transmitted from yellowed to healthy *N. rustica* plants by mechanical inoculation methods that are quite suitable for the transmission of tobacco mosaic disease to plants of this species. The failure of aster yellows to pass by mechanical inoculation of juice to a susceptible plant to which tobacco mosaic is readily passed shows how differently two virus diseases may behave in the same host.

PEACH (*PRUNUS PERSICA* STOKES)

Experiments dealing with attempts to transmit aster yellows to the peach have already been reported (4). During the past four years a number of further attempts were made to transmit the disease by both the aster leafhopper and the transplantation of yellowed aster tissues.

The experiments in which leafhoppers were used do not differ from those previously reported except that the insects were forced to feed alternately on peach and aster seedlings. The peach is apparently poisonous to the aster leafhopper. It begins to get sick on the second day after confinement on peach seedlings. By the fifth day practically all are dead. If, however, insects that have fed for two days on peach seedlings are permitted to feed on aster plants for two days they recover from the effects of



FIGURES 45-50. Aster yellows disease on various species. FIG. 45. A yellowed tomato plant bearing many short leafy branches. The disease was transmitted to this plant by the insertion of a yellowed bud of *Nicotiana rustica* L. in the stem a short distance above the ground level. The slender branching shoot produced by this bud is shown in the picture. FIG. 46. A healthy and a yellowed plant of *Potentilla monspeliensis* L. The yellowed plant is badly dwarfed. FIGS. 47 and 48. Side and front views of yellowed aster shoots growing in the stems of healthy peach seedlings. The shoots have grown from buds inserted about two months before the picture was taken. FIG. 49. A branch from a yellowed tomato plant showing the abnormal production of secondary shoots. FIG. 50. A leaf from a yellowed tomato plant showing the production of shoots in the axils of leaflets.

feeding on the peach. By alternating these two plants it is possible to keep the insects alive over their normal life period and to obtain vigorous feeding on the peach. Strong colonies bearing the virus of aster yellows were forced to feed on healthy peach seedlings for seven periods of two days each, or a total of 14 days. Using the same method, other colonies were forced to feed for many days on peach seedlings having the peach yellows disease and then on healthy peach seedlings. The results were in all cases the same. The aster leafhopper is apparently unable to transmit either aster yellows or peach yellows to the peach.

It was thought that it might be possible to transmit aster yellows to the peach by means of tissue transplantations. Healthy peach seedlings were budded with buds from yellowed aster plants. The aster buds lived for a surprisingly long period of time. Many of them lived for as long as 60 days in the peach. Some of the buds grew and produced short yellowed shoots as are shown in Figures 47 and 48. Cross sections through such aster buds show that the aster tissues united closely with the tissues of the peach. However, none of the buds lived indefinitely and most of them began to show signs of deterioration in from one to two months. In no case was aster yellows transmitted. Healthy aster buds were also transplanted to peach seedlings. They lived no longer than the yellowed buds.

In a similar manner both healthy and yellowed peach buds were transplanted to healthy aster plants. Some of both the healthy and the yellowed buds lived for more than a month but they did not in any case live as long or grow as much as the aster buds in the peach. Peach yellows was not transmitted to the aster. The evidence obtained in these experiments indicates that aster yellows cannot be transmitted to the peach and that peach yellows cannot be transmitted to the aster.

It is, however, recognized that grafting plants belonging to different families may not give favorable conditions for the transmission of virus diseases like aster yellows and peach yellows since the transplanted tissues do not live indefinitely. A protoplasmic union between cells of the stock and scion by means of the plasmodesmen probably does not occur. But such a union may be necessary for transmission.

At the time the above described experiments were under way aster yellows had not been transmitted to any species in the Rosaceae. The disease was recently transmitted to the rosaceous species *Potentilla monspeliensis*. It is planned to use this species in further attempts to transmit aster yellows to the peach.

#### CELERY (*APIUM GRAVEOLENS* L.)

In the spring of 1925 an effort was made to transmit aster yellows to the celery variety Giant Pascal. Four healthy young potted seedlings were exposed for six days to a strong colony of virus-bearing adults of *Cicadula sexnotata*. Four healthy young aster plants were exposed to the same insect

colony for the same period of time. *Cicadula* prefers the aster. Nevertheless many of the insects were observed to feed on the celery plants. Two celery plants and two aster plants were kept free from insects and served as checks. All of the four aster plants exposed to insects took yellows. All other plants remained healthy during the period of five months that they were kept under observation. From this experiment it was concluded that the celery variety Giant Pascal is either immune or highly resistant to aster yellows. Since no disease resembling aster yellows was observed on field-grown celery of other varieties, it was further concluded that they too must be resistant to the disease.

Late in 1927 a California student who visited Yonkers reported that a yellows disease of celery is the cause of serious losses to celery growers in California.

In a further attempt to transmit aster yellows to celery the following experiment was undertaken. Celery seed of the varieties Golden Plume, Henderson's Rosy Plume, Henderson's White Plume, Henderson's New Rose, Henderson's Easy Blanching, Silver Self Blanching, White Winter Queen, Golden Self Blanching, and Giant Pascal was sown early in January 1928. The seedlings were transplanted frequently enough to keep them in good growing condition. The number of plants of each variety used in this experiment and the dates on which they were exposed are shown in Table II. Two plants of each variety were kept free from insects and served as checks. All plants were exposed to the same colony which at the time the experiment was started numbered more than 1000 individuals. These insects had been reared on yellowed aster plants. Some of the leafhoppers died during the course of the experiment but the colony was still large on May 4, when the last exposure was ended. Healthy young aster plants were exposed with each set of celery plants but they were never exposed for as long a period as were the celery plants. The insects were forced to feed on each set of celery plants for at least two days. The first set of plants, as is shown in Table II, was exposed to virus-bearing insects for a period of only four days. This was due to the fact that the plants were small in March when the tests were started. As they grew larger they were exposed for longer periods of time. All plants were kept under observation for three months after the last exposure was made. All aster plants exposed to this colony of insects took yellows. All celery plants remained healthy in appearance.

It was thought that although the celery plants showed no symptoms of yellows they might, nevertheless, carry the disease in a masked form. In order to test this possibility a virus-free colony was forced to feed for one week on the 31 exposed celery plants used in the above described experiment. The insects were then caged on healthy aster plants for a period of more than a month. The aster plants remained healthy. The experiment



TABLE II  
THE EXPOSURE OF CELERY VARIETIES TO VIRUS-BEARING LEAFHOPPERS

Varieties	Exposed from Mar. 17-21, 1928 4 days	Exposed from Mar. 21-30, 1928 9 days	Exposed from Mar. 30-Apr. 6, 1928 7 days	Exposed from Apr. 6-12, 1928 6 days	Exposed from Apr. 12-19, 1928 7 days	Exposed from Apr. 19-May 4, 1928 15 days	Not exposed	Number of plants taking yellows
Golden Plume.....	1	1		1			2	None
Henderson's Rosy Plume....			1				2	"
Henderson's White Plume....	1	1	1			2	2	"
Henderson's New Rose.....			1				2	"
Henderson's Easy Blanching..	1	1			2		2	"
Silver Self Blanching.....			1		1		2	"
White Winter Queen.....	1	1		1		1	2	"
Golden Self Blanching.....	1	1	1	1		1	2	"
Giant Pascal.....	1	1			3	1	2	"

failed to bring evidence that the celery plants were symptomless carriers of the disease.

Early in 1929 Severin's (9) paper describing celery yellows and reporting his transmission experiments appeared. He obtained transmission to the varieties Easy Blanching, Giant Pascal, Golden Self Blanching, and White Plume. Celery yellows was transmitted from celery to the aster, where it is said to have produced the symptoms of aster yellows.

Severin apparently experienced no difficulty in transmitting aster yellows to celery by means of *Cicadula sexnotata*. Using much smaller colonies of insects than those used by the writer, he obtained successful transmission of the disease to 31 out of 42 plants exposed in one experiment and to 56 out of 71 plants exposed in another experiment.

Some further transmission experiments were, therefore, undertaken by the writer in 1929. Thirteen healthy young potted celery plants of the variety Golden Plume and nine similar plants of the variety Giant Pascal were exposed to a large colony of virus-bearing leafhoppers for a period of six days from June 25, 1929 to July 1, 1929. Seven plants of the variety Golden Plume and five of each of the varieties Silver Self Blanching and White Plume were exposed to virus-bearing insects for a period of five days from June 19, 1929 to June 24, 1929. Four healthy young aster plants were exposed with each set of celery plants. Four other aster plants of the same lot were kept free from insects and served as checks on the exposed plants. Four celery plants of the variety Giant Pascal and eight of each of the varieties Silver Self Blanching and White Plume were kept free from insects and served as checks for the exposed celery plants. By July 15, 1929 all of the eight exposed aster plants had aster yellows. The check aster plants



and all of the celery plants appeared to be healthy. On this date the exposed celery plants and all of the check celery plants, except one plant of each variety which was held in a greenhouse free of leafhoppers to serve as a check, were planted out in a garden in an area adjacent to a plot of asters in which there were many yellowed plants and on which large numbers of *Cicadula sexnotata* were feeding. As the season progressed the aster planting became badly yellowed. No signs of disease were observed on any of the nearby celery plants. On October 15, 1929, three months after they were planted out, all of the celery plants were dug and carefully examined. They all appeared to be normal and entirely free of any of the symptoms of aster yellows. They had grown to be large. Two experimentally exposed plants of each variety were placed in pots and transferred from the garden to a greenhouse where they were kept under observation until early in January 1930. At this time they appeared to be healthy and were discarded.

In another experiment three healthy young celery plants of each of the varieties Golden Plume, Silver Self Blanching, Giant Pascal, and White Plume were exposed to a strong colony of virus-bearing leafhoppers for a period of approximately one month, from August 3, 1929 to September 4, 1929. An equal number of plants of each variety were kept free from insects and served as checks. All plants were held under observation until October 15, 1929 when the experiment was ended. At no time did they show symptoms of yellows.

Some further transmission experiments with celery were carried out in 1930. They need not be reported as the results were the same as those obtained in the experiments already described. Aster yellows was not transmitted to the celery varieties tested under conditions that are favorable for its transmission to the aster and to many other plants.

#### ZINNIA (*ZINNIA ELEGANS* JACQ.)

In his paper on a yellows disease of celery, lettuce, and other plants Severin (9) reports the transmission of aster yellows to *Zinnia elegans*. A disease was transmitted from yellowed zinnia plants received from San Gabriel, California to aster plants. Severin reports finding a bed of zinnias in front of the Spreckels Agricultural Experiment Station which showed 100 per cent yellows. The zinnia disease was also transmitted from these plants to asters. It caused symptoms similar to those of aster yellows.

The writer has not succeeded in taking aster yellows to *Zinnia elegans*. Yellowed zinnias have not been observed in flower gardens or received from growers. Aster yellows was transmitted to *Zinnia multiflora* as is reported in another place in this paper.

An attempt was made to transmit aster yellows to *Zinnia elegans* in the summer of 1925. Six healthy young potted plants were exposed to a strong colony of virus-bearing insects for a period of nine days. Six other plants

were kept free from insects and served as checks. All plants were held under observation for five months. They all remained healthy.

In 1926 a large plot of zinnias was grown adjacent to experimental aster plots. Seven varieties were represented in this planting. They were grown from seed obtained from Peter Henderson and Company of 35 and 37 Cortlandt Street, New York, New York, and listed in the 1924 catalog of this company under numbers 4612, 4614, 4615, 4616, 4622, 4624, and 4630. The planting was kept under observation throughout the season. Although nearby aster plots were badly affected by yellows the zinnia plants remained healthy. Late in the season two plants were observed to be slightly chlorotic. It was thought that this might possibly be due to the aster yellows disease. The plants were placed in pots and brought into a greenhouse. Virus-free insect colonies failed to obtain the virus of aster yellows from the plants.

In 1928 the following varieties of *Zinnia elegans* were grown near experimental aster plots: Buttercup, Dream, Exquisite, Meteor, Oriole, and Polar Bear. The plants were kept under observation throughout the season. They remained free of the symptoms of aster yellows. The aster plots were badly affected.

Shortly after the publication of Severin's paper another attempt was made to transmit aster yellows to *Zinnia elegans*. Ten healthy young potted zinnia plants and four healthy young potted aster plants were exposed to a large colony of virus-bearing leafhoppers for a period of 11 days from July 23, 1929 to August 3, 1929. Five zinnia plants were kept free of insects and served as checks. All of the four aster plants took yellows. All of the zinnia plants remained healthy up to October 15, 1929 when the experiment was ended.

Zinnias were again planted near experimental aster plots in 1930. The plants were held under observation throughout the season. They remained free of yellows. Many of the nearby aster plants took the disease.

A number of different flower garden plots of *Zinnia elegans* were examined during the summers of 1929 and 1930. No plants were found that showed symptoms which would bring this species under suspicion of being a host of aster yellows.

However, negative evidence is never quite convincing. The writer does not wish to maintain that either *Zinnia elegans* or *Apium graveolens* are immune to aster yellows as transmitted by the leafhopper *Cicadula sexnotata*. He does maintain that these species if not immune are highly resistant to infection and that they do not readily take the disease under conditions that are favorable for its transmission to asters and to a large number of other host species.

Several visitors from California have reported that aster yellows is less severe there than it is in New York State and in some other eastern states.

In their report on California plant diseases in 1911 Smith and Smith (13) state that aster yellows is not very serious in California. Much of the aster seed for the seed trade was formerly grown in New York. Now most of the aster seed produced in this country is grown in California. The change has been attributed to the fact that aster yellows is very destructive in New York. Under these circumstances it is difficult to understand how zinnias at the Spreckels Agricultural Experiment Station could become 100 per cent infected while those grown near badly yellowed aster plots at Yonkers, New York, remain 100 per cent free from infection. It is, of course, possible that aster yellows behaves differently under the climatic conditions of California than it does in the eastern states. It is also possible that the yellows with which Severin has been working in California is not aster yellows. Further experiments will no doubt yield the correct explanation.

#### DISCUSSION

No new symptoms were discovered in studies on the new hosts. The symptoms previously described recur with surprising regularity. There are, however, many plants that do not show all of the symptoms characteristic of yellows on the China aster. Although chlorosis is a symptom of the disease in many plants, there are a few, like *Levisticum paludapifolium* and *Coreopsis lanceolata*, that have shown no chlorosis whatever under the conditions of the writer's experiments. There are a number of plants in which chlorosis is slight. This is characteristic of hosts in the families Carophyllaceae, Cruciferae, and Labiatae. Several species in the Solanaceae also show a mild form of chlorosis. Yellows has been transmitted to only one species in each of the families Onagraceae, Begoniaceae, Polygonaceae, Apocynaceae, and Recedaceae. It does not bring about much chlorosis in any of these species. It causes marked chlorosis in most of the host species of other families. Yellows does not completely suppress chlorophyll production in any of the species studied. Symptoms are not known to be entirely masked in any species. No plant has ever been observed to recover. The yellows form of chlorosis is typical for the disease. Plants never produce white leaves or branches as a result of aster yellows.

In some species aster yellows may entirely prevent the formation of flowers. In others it causes the production of virescent flowers and of interesting malformations of the reproductive organs. Its effect on the flowers depends very largely on the age of a plant at the time it takes yellows. A few species produce approximately normal colored flowers even after having the disease for many months. One of the best examples of such tolerance is found in *Begonia semperflorens* Link and Otto. The disease causes sterility, however, in the tolerant as well as in the nontolerant species. Seeds from diseased plants or from diseased branches of partly diseased plants are not viable.

The abnormal production of secondary shoots is apparently a constant symptom of aster yellows. It has appeared in all plants that were held under observation for a sufficient period of time. It results from the stimulation of buds that should remain dormant. So far as is known, the disease does not cause the production of adventitious buds. The leafy shoots that grow from the axils of leaflets of yellowed tomato plants and from the pistils of the flowers of various species as a result of yellows infection probably arise from bud primordia that are normal for the healthy plants of these species. The fact that the shoots always arise at exactly the same point in the axils of tomato leaflets and in the terminal portions of flower pistils seems to support this view.

Another equally constant symptom results from the effect of the disease on the response of plants to gravity. Instead of the branches taking positions that are normal for the several species, they assume a more upright habit of growth. This symptom is conspicuous in most of the species studied. There are a few, however, in which it is not very noticeable. The habit of growth of diseased *Clarkia elegans* plants is not greatly different from that of healthy plants. The diseased branches do not take the vertical position which is so characteristic for the branches of most yellowed plants.

Stunting is a striking symptom of yellows in some species. Certain plants, like the tomato and the petunia, grow very little after taking the disease. Others, like *Asclepias nivea* L. and *Begonia semperflorens*, grow for an indefinite period of time and are not much stunted. Between these extremes there are many different degrees in the effects of yellows on growth. The plants of most species are severely stunted if they become infected while young.

Clearing of veins in one or more leaves or parts of leaves is the earliest symptom of yellows in a number of species. It seems to be a transient symptom which accompanies the first onset of the disease. It does not appear distinctly in any of the new leaves after the first onset. It is not a symptom of yellows in most plants.

Diseased plants of many species die prematurely. It is believed, however, that this is not a direct result of the disease. Yellowed plants are more susceptible than healthy ones to attack by various fungi such as species of *Botrytis* and *Alternaria*. It is these fungi that seem to kill infected plants. The yellows disease does not cause extensive necrosis in any species studied. Through the stimulation of growth in secondary shoots it causes the production of large numbers of very succulent shoots. Such shoots are slender and usually lacking in stiffness. They are frequently unable to support the weight of the leaves and branches they bear. As the abnormal growth develops they may become top-heavy and fall over. This results in mechanical injuries that lead to death in some instances.

Resistance of plants to infection does not seem to be associated with

tolerance for the disease. Some of the species most resistant to infection are most severely injured. *Petunia hybrida* is highly resistant but is one of the least tolerant to yellows of all the plants studied. *Clarkia elegans*, on the other hand, is quite susceptible to infection but shows a high degree of tolerance. Plants which, like the China aster, have a low degree of tolerance and a high degree of susceptibility to infection are the ones most severely damaged by yellows. Species which, like *Begonia semperflorens*, are both resistant to infection and tolerant to the disease, are not greatly injured.

The characteristics of aster yellows as a disease cannot be adequately studied in any one species. It is necessary to know it in a number of different hosts in order to gain a clear idea of its symptoms and effects. It differs from most other virus diseases of plants in its peculiar influence on the reproductive organs. It is, in this respect, similar to the false blossom disease (12) of the cranberry (*Oxycoccus macrocarpus* Pers.).

The fact that aster yellows goes to a considerable number of different plants must not be taken to indicate that it is lacking in specificity. A number of families and a very large number of species are apparently immune.

The host range of the disease does not coincide with that of its insect vector. Many of the plants that are preferred by the insect do not take yellows. Although the leafhopper feeds readily on corn, oats, wheat, barley, rye, rice, and a large number of different grasses, it is not known to transmit yellows to any species in the Gramineae. On the other hand, it transmits yellows to certain plants that are quite toxic to it and on which it cannot live for more than a few days. *Schizanthus* and *Pyrethrum* furnish examples of this type of relationship.

Aster yellows has not yet been transmitted to a single monocotyledonous plant. Of the dicotyledonous plants tested less than 20 per cent have taken the disease. Yellows was not transmitted to any leguminous plant although many species and varieties were exposed under favorable conditions for infection. A number of species belonging in the Rosaceae were also exposed in the same manner. *Potentilla monspeliensis* is the only one to which it has been transmitted.

Approximately one-half of all the known host species belong in the Compositae. Many of the hosts in this family are highly susceptible. Some of them, however, take the disease only under favorable growing conditions and when exposed to large numbers of virus-bearing insects. Most of the composites seem to be immune to aster yellows.

It has not been possible to determine what characteristics are associated with susceptibility to yellows. Immunity, resistance, and a high degree of susceptibility are in some instances shown by closely related species. Wide variations in susceptibility may also exist between varieties

of the same species. Such relationships were noted in the genera *Centaurea* and *Plantago* (5).

It is probable that aster yellows will be found to attack many plants that have not yet been reported as hosts of the disease. Some of the species that have resisted yellows up to the present time may succumb under environmental conditions more favorable to infection than those which have obtained in past tests. Each summer's observations on wild and cultivated plants furnishes a new list of species having diseases with symptoms similar to those of aster yellows. Transmission experiments with such plants will, no doubt, add many new names to the list of susceptible species. A number of the new hosts here reported are European plants. Others would, doubtless, be discovered if a larger number of foreign species were exposed to the disease. It is possible that many species which have escaped infection after exposure to virus-bearing leafhoppers would, like the tomato, prove to be susceptible if diseased scions of related species were grafted upon them. It is also possible that some other insect vector might carry the disease to species to which it is not transmitted by *Cicadula sexnotata*.

Many of the new hosts of aster yellows are cultivated flowering plants. Some of them are weeds. A few, such as New Zealand spinach, tomato, carrot, and parsnip, are plants of some economic importance.

Most of the hosts listed in this paper belong in the 23 families already reported as having one or more susceptible species. The remainder belong in 15 other families. One hundred and three genera are represented in the new list. All but five of these are genera to which the disease had not previously been taken. Aster yellows has now been experimentally transmitted by *Cicadula sexnotata* to 170 species belonging in 38 different families of plants. It has been transmitted to one additional species by means of tissue transplantation. It is interesting that this disease, which spreads naturally to such a large number of different plants and was taken experimentally to so many different species, has not yet been transmitted mechanically by means of juice from diseased tissues to any plant. Mechanical transmission has been accomplished only by the transplantation of living tissues. Natural spread seems to be due entirely to *Cicadula sexnotata*.

The transmission of aster yellows by this leafhopper is a distinctive characteristic of the disease. While its plant host range is very wide, its insect relationships are apparently very narrow. Some other insect may eventually be found which can transmit the disease. A considerable number of species that feed and breed on the aster or other susceptible plants are certainly unable to do so.

All evidence available at present indicates that aster yellows is due to a single causative agent. If two or more independent agents combined in causing it, they should become separated occasionally in transmission from

aster to aster. Moreover, it is not to be expected that two or more viruses would have identical host ranges including so many different species of plants as are listed among the hosts of aster yellows. The disease has not yet been transmitted from all of these species to the aster and shown to produce in the aster the typical symptoms of yellows. It has, however, been transmitted to the aster from 40 different species. In some of these species it causes symptoms that are very different from those shown by yellowed aster plants. When taken from such species to the aster the symptoms produced are in all cases those of aster yellows. No other disease resembling this is known to attack the aster or any one of a number of other susceptible plants. It seems probable, therefore, that it results from the action of a single causative agent.

In view of the fact that this agent produces disease in so many different species and that in a number of species it causes little or no chlorosis, the name, aster yellows, seems somewhat inadequate. From the standpoint of plants affected it might, with almost equal appropriateness, take its name from a number of other species. From the standpoint of symptoms, stunt disease, bunchy top, or false blossom would be almost as suitable as yellows. It has, however, been known as aster yellows for many years. The name is well established in the literature of both plant pathology and floriculture. These are perhaps sufficiently good reasons for not changing it, even if a more appropriate name could be found. And likewise, until more is known of the causative agent we may with considerable justification continue to call it the virus of aster yellows.

#### SUMMARY

1. The experimental transmission of aster yellows to 120 new host species is reported. The most striking symptoms on each host plant are described.

2. The new hosts belong in 30 different families of plants. Fifteen of these are families to which the disease had not previously been transmitted. The following families are represented by the hosts here reported: Caryophyllaceae, Moraceae, Chenopodiaceae, Nyctaginaceae, Aizoaceae, Ranunculaceae, Papaveraceae, Cruciferae, Rosaceae, Limnanthaceae, Datisceae, Cistaceae, Loasaceae, Onagraceae, Umbelliferae, Plumbaginaceae, Primulaceae, Apocynaceae, Polemoniaceae, Hydrophyllaceae, Boraginaceae, Labiatae, Solanaceae, Scrophulariaceae, Gesneriaceae, Acanthaceae, Plantaginaceae, Valerianaceae, Campanulaceae, and Compositae.

3. Aster yellows was not transmitted mechanically by means of juice from diseased tissues to any plant. The disease was carried to all host plants except the tomato by means of *Cicadula sexnotata*. It was transferred to the tomato by budding.

4. Some other plants to which aster yellows was not transmitted after repeated exposures to virus-bearing colonies of *Cicadula sexnotata* are: potato, tobacco, peach, celery, and *Zinnia elegans*.

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# THE INHIBITING EFFECT OF OXIDASE ON THE REDUCTION OF SULPHUR BY POTATO AND GLADIOLUS JUICE<sup>1</sup>

JOHN D. GUTHRIE

When dormant potato (*Solanum tuberosum* L.) tubers are treated with ethylene chlorhydrin, a large change in the reducing action of the juice of these tubers is noted after several days. This change in reducing power is shown by an increase in the ability of the juice to reduce methylene blue, iodine in acid solution, and phosphotungstic reagents. This effect has been reported in a previous paper (2). Subsequently it has been found that treatment with ethylene chlorhydrin results also in an increase in the power of the juice to reduce elementary sulphur to hydrogen sulphide at ordinary temperatures. It has also been found that ethylene chlorhydrin treatment of corms of *Gladiolus*, variety "Souvenir", results in an increase in the power of the juice to reduce sulphur. The production of hydrogen sulphide from sulphur by the action of a plant extract was first observed by de Rey-Pailhade (6) who used an alcoholic extract of yeast. It is a reaction of especial interest, since it is characteristic of sulphydryl compounds such as glutathione and cysteine (4).

In order to see if the reduction of sulphur by the juice was enzymic, the effect of boiling the juice was studied. The results were just opposite to what would be expected if the reaction was aided by an enzyme. It was found that boiling the juice resulted in an increased production of hydrogen sulphide. The experiments described in this paper were made for the purpose of explaining this difference between the action of boiled juice and the action of unboiled juice. It will be shown that the presence of oxidase in the unboiled juice is responsible for the difference.

## EXPERIMENTS

The method of McCallan and Wilcoxon (5) has been used in studying the reaction. Their method consists of placing sulphur paste in the bottom of a wide-mouthed bottle, mixing the material to be tested with it, and inserting a rubber stopper from which is suspended a piece of basic lead acetate paper. The rate of hydrogen sulphide production is estimated by noting the time necessary for the test paper to reach a standard color.

Juice was obtained by grinding the gladiolus corms or peeled potato tubers through the butter-nut cutter of a food chopper, squeezing through cheesecloth, and centrifuging. Boiled juice was prepared by placing a test tube of the centrifuged juice in boiling water for ten minutes, cooling in cold water, and then filtering off the coagulum.

The results of boiling the juice of potato tubers and the juice of gladi-

<sup>1</sup> Herman Frasch Foundation for Research in Agricultural Chemistry, Paper No. 19.

olus corms that have received various ethylene chlorhydrin treatments are shown in Table I. Five cc. of juice were used in each case and the production of  $H_2S$  was completed in 3 hours in the case of gladiolus juice and in 1.5 hours in the case of potato juice. From Table I it is evident that boiling results in a marked increase in hydrogen sulphide production.

TABLE I  
THE EFFECT OF BOILING UPON THE SULPHUR REDUCTION BY JUICE OF TREATED  
AND UNTREATED POTATO TUBERS AND GLADIOLUS CORMS

Treatment	Total $H_2S$ produced mg.	
	Fresh	Boiled
Potato—24 hr. dip treatment—30 cc. of 40% ethylene chlorhydrin per l.	.057	.097
Potato—24 hr. dip treatment—10 cc. of 40% ethylene chlorhydrin per l.	.000	.036
Potato—24 hr. dip treatment—Water.....	.000	.000
Gladiolus—3 day vapor treatment—3 cc. of 40% ethylene chlorhydrin per l.	.037	.083
Gladiolus—1 day vapor treatment—1 cc. of 40% ethylene chlorhydrin per l.	.018	.056
Gladiolus—untreated.....	.000	.026

Two explanations for the effect of boiling presented themselves. One was that some substance was formed by the process of boiling, possibly sulphhydryl compounds split off from proteins; the other, that there was a competition between sulphur and oxygen for the hydrogen donors that are responsible for the sulphur reduction. Dialyzed potato juice which should contain the proteins of the original juice was boiled, but showed no power to reduce sulphur after this treatment. Since other methods of testing the first hypothesis were not evident, attention was turned to the second.

If competition between oxygen and sulphur is involved, the presence of oxidase in the fresh juice would favor the oxygen and hence explain the low yield of hydrogen sulphide from fresh juice. If this were true, addition of fresh juice to boiled juice should lower the hydrogen sulphide production from the latter. This was tried and the results are shown in Table II. It

TABLE II  
THE EFFECT OF ADDING FRESH JUICE TO BOILED JUICE

Material used	Total $H_2S$ mg.
5 cc. boiled juice of treated potatoes + 5 cc. $H_2O$ .....	.085
5 cc. boiled juice of treated potatoes + 5 cc. of fresh juice of untreated potatoes	.000
5 cc. boiled juice of treated gladiolus + 5 cc. $H_2O$ .....	.066
5 cc. boiled juice of treated gladiolus + 5 cc. of fresh juice of untreated gladiolus	.016

will be seen that the addition of fresh juice to boiled juice results in a decrease in hydrogen sulphide production. This decrease is greater in the case of potato than in the case of gladiolus. This is in agreement with the oxidase content of the two juices. Estimated by a method that has been

previously described (3), potato juice has about five times as much oxidase activity as gladiolus juice. As additional evidence of the effect of adding oxidase to boiled juice, boiled and unboiled dialyzed potato juice was added to boiled gladiolus juice. The results are shown in Table III. The potato juice was dialyzed overnight and diluted to one-third of its original concentration.

TABLE III  
THE EFFECT OF ADDING DIALYZED POTATO JUICE TO BOILED GLADIOLUS JUICE

Material used	Total H <sub>2</sub> S produced mg.
5 cc. of boiled, treated gladiolus juice + 5 cc. boiled, dialyzed potato juice. . . .	.040
5 cc. of boiled, treated gladiolus juice + 5 cc. of unboiled dialyzed potato juice.	.008

In order to show that oxygen is involved in the inhibition, the reaction was tried in an atmosphere of nitrogen. Unboiled juice was placed in a vial that could be tilted so as to mix it with the sulphur. The stopper of the bottle was fitted with a stopcock so that it could be evacuated. This was done and the bottle filled with nitrogen that had been purified by passing over hot copper, prepared by the reduction of copper oxide wire with hydrogen. The bottle was evacuated and filled with nitrogen several times. The reaction was started by tipping the vial and the darkening of the lead acetate paper was then noted with the following results:

	H <sub>2</sub> S test
2 cc. of fresh, treated potato juice in nitrogen	+
2 cc. of fresh, treated potato juice in air	—

This was repeated several times with the same results. Nitrogen gas directly from the tank was also effective.

It was considered of interest to find out whether the fresh juice prevented the formation of hydrogen sulphide, or whether it would destroy the hydrogen sulphide already formed. Therefore, the action of potato juice on hydrogen sulphide was studied by adding NaSH to potato juice, allowing it to stand, and then testing for hydrogen sulphide. One cc. each of water, boiled juice, and fresh juice was placed in separate bottles, 2 cc. of NaSH solution containing 5 mg. per liter were added to each, stoppered, and allowed to stand five minutes. New stoppers carrying lead acetate paper were quickly inserted and the color observed with the following results:

	H <sub>2</sub> S test
Water	+
Boiled juice	+
Fresh juice	—

This was repeated several times with the same result. In order to avoid removing the stoppers to insert the lead acetate paper, the experiment was performed in a closed system. The results were the same. This experiment shows that fresh juice acts to destroy sulphides, probably by accelerating their oxidation by oxygen.

#### DISCUSSION

It will be seen from the above experiments that boiling or carrying out the reaction in a nitrogen atmosphere results in an increase in the power of the juice to reduce sulphur, while the addition of fresh or dialyzed juice to boiled juice brings about a decrease in the power to reduce sulphur. These results may be explained by the presence of oxidase in the fresh or dialyzed juice. In the fresh juice, the action of oxygen and oxidase on the hydrogen donors responsible for the reduction of sulphur and on the hydrogen sulphide as it is formed causes a low hydrogen sulphide production. Boiling destroys oxidase which is necessary for the action of oxygen, but not for the action of sulphur. Therefore, in the boiled juice the action of the hydrogen donors is on the sulphur, producing hydrogen sulphide. Removal of oxygen, even though oxidase is present, stops oxidation and, consequently, leads to an increased hydrogen sulphide production. When fresh or dialyzed juice is added to boiled juice, oxidase is introduced and its inhibiting action is manifested by a decrease in hydrogen sulphide production. Thus, there is a competition between oxygen and sulphur, and oxidase exerts its effect by increasing the activity of the oxygen. When the action of oxygen predominates, the product of the reaction is chiefly water; when the action of sulphur predominates the product of the reaction is principally hydrogen sulphide.

It is not to be inferred that boiling will always result in an increased hydrogen sulphide production. Obviously the effect would not be observed in the absence of oxidase. Furthermore, there seems to be a difference between extracts and tissues in this respect. Abelous and Ribaut (1) found that heating liver extracts resulted in an increased hydrogen sulphide production, while Sluiter (7) found that heating liver suspensions to 70° C. decreased their power to reduce sulphur, and McCallan and Wilcoxon (5) showed that reduction of sulphur by fungous spores had a temperature optimum of 35° C. Since the temperature effects in the present work differed from those found in the experiments of Sluiter (7) and of McCallan and Wilcoxon (5) an explanation is desirable. A probable one is that cells have the power to act on RS·SR to reduce it back to RSH. This regenerative action of tissues was shown by Hopkins (4) in his first paper on glutathione. Since McCallan and Wilcoxon find that the production of hydrogen sulphide is much greater than can be explained by the sulphydryl content of the spores, it is suggested that a regenerative mechanism is present in

the spores. Sluiter's data (7) show that the hydrogen sulphide production of suspensions of animal tissues is frequently greater than that to be expected from their sulphhydryl content. A regenerative mechanism may therefore be involved. An adequate explanation for the inhibiting effect of heating in these two cases is that it destroys the regenerative action of the cells and therefore stops the formation of more sulphhydryl compounds. In the juices used in the present work, regenerative action is either absent or present to such a small extent as to be obscured by the marked effect of oxidase. To state the case so as to include the results described in the present paper and the results obtained by other workers, it may be said that boiling, by inactivating all enzymes, tends to increase hydrogen sulphide production by stopping the action of oxidase and oxygen on the sulphhydryl compounds already present, but it also tends to decrease hydrogen sulphide production by stopping the formation of more sulphhydryl compounds. Therefore, the amount of hydrogen sulphide produced and the effect of boiling will depend on which of these two actions predominates.

#### SUMMARY

Treatment of dormant potato tubers or gladiolus corms with ethylene chlorhydrin results in an increase in the power of the juice to reduce sulphur to form hydrogen sulphide. This action is increased by boiling the juice or carrying out the reaction in a nitrogen atmosphere. Addition of unboiled juice to boiled juice results in a decrease in hydrogen sulphide production. Fresh juice catalyzes the oxidation of hydrogen sulphide. These results are explained by the presence of oxidase in the unboiled juice. In the presence of oxygen, the oxidase exerts an inhibiting effect by catalyzing either the oxidation of the hydrogen donors responsible for the reduction of sulphur or the oxidation of hydrogen sulphide as it is formed. An explanation is given for apparent inconsistencies in the literature dealing with the effect of heat on the power of tissues and extracts to reduce sulphur.

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## MOSAIC DISEASE OF TOBACCO. II. ACTIVITY OF THE VIRUS PRECIPITATED BY LEAD ACETATE

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### INTRODUCTION

In a previous paper by Vinson and Petre (13) it was reported that the virus of mosaic disease of tobacco had been freed of 90 per cent of the accompanying solids of the juice without apparent loss of infective power. Evidence is now submitted to show that the total solids of the virus fraction have been reduced to about one per cent of that of the original material without reducing the infective power.

The partial purification reported by Vinson and Petre (13) was accomplished by first clearing the juice from frozen diseased plants of *Nicotiana tabacum* L., var. Turkish with basic lead acetate solution, then precipitating the virus at low temperature with acetone. It was soon learned, however, that the virus fraction thus prepared still contained a large amount of irrelevant material. Much of this fraction consists of the calcium salts of organic acids, and these are difficult to remove directly from the fraction without losing virus at the same time. Removal of calcium from the juice with oxalate causes loss of infectivity; and fractional precipitation with acetone, at low temperature, is unsatisfactory. The amount of solids in this fraction may be further reduced by adding acid until the hydrogen ion concentration of about pH 4.5 is reached, then precipitating with acetone at low temperature. The precipitate thus obtained is very light in color as most of the pigment remains in the supernatant liquid. Highly infectious solutions are obtained by redissolving the precipitate in water. Preparations containing less than 10 per cent of the solids of the original juice have been obtained in this way. In the presence of acetone the time of subjection to high hydrogen ion concentration must be short (5 to 10 min.) if activity of the virus is to be preserved. Juice from diseased plants has been observed to retain activity at pH 1.8 to 1.9, when no acetone was added. Complete loss of activity was observed, however, when a juice sample was brought to pH 2.5, then precipitated with two volumes of acetone at room temperature.

It is possible to throw out of solution an active fraction by acidifying a solution of the acetone precipitate. Precipitation of the virus is never complete, however, at any reaction which the virus will endure. An active fraction is also thrown out of juice from diseased plants at alkaline reaction. The precipitation in this case also is not complete at any reaction which the virus will endure. Down to a hydrogen ion concentration equivalent to pH 9.1 to 9.2 addition of alkali causes no more striking effect than



the addition of acid. At pH 9.2 to 9.5 an abrupt decrease in activity occurs. The activity of a virus solution at pH above 9.2 corresponds to that of a juice sample from diseased plants diluted 1 to 10,000. Inactivation at alkaline reaction has been observed not to go to completion in 24 hours at pH 10.5. Alkaline inactivation may be arrested by bringing the solution to acid reaction, but the addition of acid does not restore the activity of solutions inactivated above pH 9.2. Precipitation of the virus with acetone from juice at alkaline reaction has not been practiced since this throws down more of the pigment.

## RESULTS

### RESULTS WITH THE SAFRANIN PRECIPITATE

The active dispersion of the precipitate obtained on adding a dilute safranin solution to juice from diseased plants (13) contains 7 to 8 per cent of the original solids. It contains very little or no calcium, which is so difficult to eliminate from the acetone precipitate. The safranin precipitate of the virus may be decomposed with amyl alcohol (13) and a highly infectious solution, very low in solids, obtained. Solutions have been obtained through the safranin procedure which approximated the original juice in infective power. In all such solutions, however, brown pigment was present, even though the juice had been cleared with basic lead acetate previous to precipitation with safranin. On concentrating these solutions the brown pigment becomes so troublesome that hope of obtaining a good product from this fraction has been abandoned for the time being.

### PRECIPITATION OF THE VIRUS WITH A SOLUTION OF LEAD ACETATE

In clearing the juice with a solution of basic lead acetate it was recognized that this reagent could be used only within certain limits of concentration without causing loss of activity in the supernatant liquid. It was at first supposed that this was due to destruction of virus by the reagent. On testing the various fractions for activity, however, the virus was found to be precipitated, and was found in an active form in the lead precipitate. The supernatant liquid, obtained on clearing with 15 cc. basic lead acetate solution per 500 cc. of juice (13), when treated with 35 cc. additional basic lead acetate per 500 cc. of the juice, gives a cream colored precipitate. The supernatant liquid from the second precipitate is low in activity, while a suspension of the precipitate is highly infectious as shown in Table I. The data in this table show that a greater percentage of the plants, inoculated with the suspension, became diseased than in the case where juice from diseased plants was used. This greater infective power is in accord with increased activity observed in the presence of suspended material (p. 143).

To free the virus from the lead precipitate the precipitate was first decomposed with hydrogen sulphide. The supernatant liquid from the lead

TABLE I  
COMPARISON OF THE INFECTIVE POWER OF A SUSPENSION OF THE LEAD PRECIPITATE  
OF THE VIRUS WITH THAT OF THE ORIGINAL JUICE

Lead precipitate of the virus, after acid phosphate elution, suspended in a volume of distilled water equal to the original sample of juice		The corresponding juice from diseased plants, undiluted	
No. of plants inoculated*	No. of plants diseased	No. of plants inoculated*	No. of plants diseased
10	9	10	8
10	9	10	8
10	10	10	9
10	10	10	6
10	10	10	6
10	10	10	6
10	8	10	6
10	8	10	9
10	10	10	9
10	8	10	9
10	10	10	7
10	9	10	7
10	10	10	7
10	9	10	7
10	8	10	7
10	7	10	7
10	10	10	7
10	8	10	7
10	8	10	7
10	9	10	7

\* Plants were inoculated by puncturing 4 leaves of each plant with 5 pins dipped into the inoculum; a total of 20 punctures per plant.

sulphide was only very slightly infectious. This failure to recover the virus may be due, in part, to some of the virus being carried down with the lead sulphide and to the fact that the hydrogen ion concentration rises above that at which the virus is inactivated (p. 132). Suspending the precipitate in a buffered solution of hydrogen ion concentration of about pH 6.0, then decomposing with hydrogen sulphide, gives better results, although the virus may be freed at this reaction without employing hydrogen sulphide. Attempts to recover the virus in the supernatant liquid by decomposing the lead precipitate with oxalate, sulphate, and carbon dioxide were unsuccessful. Finally it was found that the virus could be removed by suspending the lead precipitate in a M/15 potassium hydrogen phosphate solution of hydrogen ion concentration of about pH 6.5. This gives a highly infectious supernatant liquid, but one which contains organic acids. By a preliminary elution of the lead precipitate of the virus with a third molar solution of  $\text{KH}_2\text{PO}_4$ , a large part of the solids are removed and hence eliminated. The virus, however, is not removed. This preliminary treatment of the neutral lead acetate precipitate of the virus with acid phosphate solution is a very important step in our present method of further purifying the virus of mosaic disease of tobacco.

The acid phosphate apparently removes very little virus from the lead precipitate, as shown in Table II. This apparent low infectivity of the acid phosphate eluate cannot be attributed to inactivation of the virus as it is removed. A juice sample diluted with an equal volume of 0.66 molar primary potassium ortho-phosphate solution, although less infectious than the same juice diluted correspondingly with distilled water, is accompanied by some injury to the tissue on inoculation into the plant. Since a juice sample 0.22 molar in primary potassium ortho-phosphate and three times the original volume is as infectious as the original juice correspondingly diluted with distilled water, any virus eluted by  $M/3$  acid phosphate should be active in this solution. Evidence in this regard is reported in Table III.

TABLE II  
INFECTIVITY OF THE ACID PHOSPHATE ELUATE

Preparation	Dilution	No. of plants inoculated	No. of plants diseased
Original juice	Diluted with an equal volume of water	162	91
Lead precipitate of the virus eluted with a normal solution of primary potassium ortho-phosphate; eluate used to inoculate plants.	Undiluted	162	13

TABLE III  
RESULTS OF DILUTING THE ORIGINAL JUICE WITH SOLUTIONS  
OF PRIMARY POTASSIUM ORTHO-PHOSPHATE

Exp. No.	Preparation	No. of plants inoculated	No. of plants diseased
1	Original juice diluted with two volumes of distilled water	162	97
	Original juice diluted with two volumes of $1/2$ M primary potassium ortho-phosphate solution	161	59
2	Original juice diluted with two volumes of distilled water	159	94
	Original juice diluted with two volumes of $M/3$ primary potassium ortho-phosphate solution	162	99

The possibility that the  $M/3$  primary potassium ortho-phosphate solution removes virus from the lead precipitate but shows low activity due to inactivation is precluded by the experiments reported in Table IV. In each of these experiments neutral lead precipitates of the virus were simultaneously prepared from the same juice sample, then the lead precipitate was eluted in one case with water and in the other with a  $M/3$  solution of pri-

mary potassium ortho-phosphate. The results indicate clearly that as compared with a water wash as a control,  $M/3$   $KH_2PO_4$  elution is certainly not inactivating. The purification obtained by elution of pigment by  $M/3$   $KH_2PO_4$  is accompanied by no significant loss in virus either by elution or inactivation and is, therefore, a gain in relative virus concentration.

TABLE IV

COMPARISON OF THE EFFECT OF A NORMAL SOLUTION OF  $KH_2PO_4$  ON THE VIRUS IN THE NEUTRAL LEAD PRECIPITATE WITH DISTILLED WATER AS CONTROL

Exp. No.	Preparation*	Plants inoculated	Plants diseased	Likelihood of a difference (a) vs. (b) or (c)	Per cent of original virus	Likelihood of a difference (c) vs. (b)
1	(a) Original juice sample	159	94			
	(b) $KH_2-K_2HPO_4$ pH 6.5 eluate of the lead precipitate. No previous $KH_2PO_4$ elution	162	79	109:1	40	
	(c) $KH_2-K_2HPO_4$ pH 6.5 eluate of the lead precipitate following elution with $M/3$ $KH_2PO_4$	161	93		99	71:1
2	(a)**	161	80			
	(b)	155	58	12,500:1	22	
	(c)	162	71	24:1	66	54:1
3	(a)	162	102			
	(b)	159	75	$> 10^4:1$	18	
	(c)	158	66	$> 10^4:1$	10	9:1
4	(a)	160	139	$> 10^4:1$		
	(c)	162	112	$> 10^4:1$	35	
5	(a)	158	107			
	(c)	162	112	3:1	115	

\* All the above preparations were inoculated at a dilution of 1 to 3. Juice samples were diluted with two volumes of distilled water, eluates were diluted with a second eluate of the residue equal to twice the volume of the first eluate.

\*\* (a), (b), (c) Experiments 2, 3, and (a), (c) of 4 and 5 were prepared in the same way as the corresponding preparations of Experiment 1.

The data in Table V show the reduction in organic solids of the neutral lead acetate precipitate accompanying acid phosphate elution. The separation of the active substance from a high proportion of irrelevant material at this step yields an active preparation with an organic solid content of 1 to 1.4 per cent of the total solids of the original juice.

#### INVESTIGATION OF THE PRECIPITATE OBTAINED ON CLEARING THE JUICE WITH A SOLUTION OF BASIC LEAD ACETATE

The loss of infective power due to virus accompanying the basic lead acetate precipitate in the preliminary clearing has been investigated by ex-

TABLE V  
THE LOSS OF ORGANIC SOLIDS ON ELUTION OF THE LEAD PRECIPITATE  
OF THE VIRUS WITH A NORMAL SOLUTION OF ACID PHOSPHATE

Exp No.	Preparation	Total organic solids in grams	Total nitrogen in grams
1	Neutral lead acetate precipitate obtained by adding 40 cc. of neutral lead acetate solution (200 grams in 1000 cc. of water) to 500 cc. of juice which had been previously cleared with 15 cc. of basic lead acetate solution (200 grams of Horne's basic lead acetate in 1000 cc. of water)	0.8100	0.0410
1(a)	Precipitate prepared as in No. 1, then eluted with a normal solution of primary potassium ortho-phosphate	0.1760	0.0260
2	Same as No. 1	0.7740	0.0460
2(a)	Same as No. 1(a)	0.1320	0.0230
500 cc. of the original juice		12.5769 total solids	0.3925

amination of the supernatant liquid and also the solution of the acetone precipitate (13) obtained from the supernatant liquid. No distinction could be made between the infective power of the original juice and the cleared juice at equal dilution. The clearing with basic lead acetate solution, therefore, did not seem to reduce the virus concentration in the supernatant liquid. Examination of the neutral phosphate eluate of the basic lead acetate precipitate (Table VI, Exp. 3) has shown that some virus is removed in clearing. When the basic lead acetate precipitate obtained on clearing is washed with successive portions of water, equal in volume to the juice sample from which the precipitate is obtained, the first wash is clear and colorless, and the succeeding washes are opalescent. Table VI, Experiments 1 and 2, give the results obtained on using the aqueous wash of the basic lead precipitate. These results show that the basic lead acetate precipitate appears to remove considerable virus. Suspended material, however, such as talc and norite, markedly increase the infective power of juice from diseased plants. The activity of the suspensions in Table VI may, therefore, offer an exaggerated estimate of the amount of virus actually present in the basic lead acetate precipitate, obtained on preliminary clearing of the juice. Table VI, Experiment 3, shows that the activity of the eluates of Experiment 2 is due to suspended material. Following elution of the virus by neutral phosphate, the suspension obtained on washing the residue of the basic lead acetate precipitate is considerably decreased in activity. Undiluted, the neutral phosphate eluate of Experiment 3 is almost as active as the original juice diluted to three times the original volume. That the basic lead acetate precipitate obtained on clearing might

possibly contain one-third the original amount of virus, or 27 per cent calculated from the equation of Holmes' (4) curve  $z = 1.2^x$  ( $z$ =dilution;  $x$ =difference in diseased plants per 50 plants) is the most liberal interpretation that can be put on these figures.

TABLE VI  
ACTIVITY OF ELUATES AND WASHES OF BASIC LEAD ACETATE  
PRECIPITATE OBTAINED ON CLEARING

Exp. No.	Preparation	Plants inoculated	Plants diseased
1	Juice diluted 1 to 3	162	97
	Second aqueous wash of the basic lead acetate precipitate (undiluted). A suspension.	158	63
2	Juice diluted 1 to 3	161	85
	Second aqueous wash of the basic lead acetate precipitate (undiluted). A suspension.	162	74
	Third aqueous wash of the basic lead acetate precipitate (undiluted). A suspension.	162	83
3	Juice diluted 1 to 3	159	94
	Neutral phosphate eluate of the basic lead acetate precipitate (undiluted).	162	91
	Aqueous wash basic lead acetate precipitate following above neutral phosphate elution (undiluted). A suspension.	162	29
(a)	Juice diluted 1 to 3	159	105
(b)	Neutral phosphate eluate basic Pb precipitate after H <sub>2</sub> O elution. In 3 times volume of the original juice.	160	79
4(c)	Neutral phosphate eluate basic Pb precipitate after acid phosphate elution. In 3 times volume of the original juice.	158	73
(d)	Neutral phosphate eluate neutral Pb precipitate after acid phosphate elution. In 3 times volume of original juice.	162	111

Basic lead acetate precipitates considerable of the brown pigment of the tobacco juice. The neutral phosphate eluate of the neutral lead precipitate following acid phosphate elution is practically free of this colloidal pigment. To determine whether the pigment in the neutral phosphate eluate of the basic lead acetate precipitate is responsible for the activity of the virus in a manner similar to augmentation of activity by suspended material, the activity of the neutral phosphate eluates of the basic lead acetate precipitate with and without acid phosphate elution, were compared. In Table VI, Experiment 4, (a) and (c) show that the activity of the neutral phosphate eluate of the basic lead acetate precipitate is not solely de-

pendent on the brown pigment. The experiment does not exclude activation or support of virus activity by the pigment.

#### THE NEUTRAL LEAD ACETATE PRECIPITATE FOLLOWING PRELIMINARY CLEARING

In Table IV a comparison is made between the activity of the original juice (a) and the mixed phosphate eluate of the neutral lead acetate precipitate following preliminary elution with acid phosphate (c). In addition to the investigation (p. 134) of possible inactivation of virus by  $\text{KH}_2\text{PO}_4$  to account for the inappreciable activity of the acid phosphate eluate in Experiments 1, 2, 3 of Table IV, parts (b) show the activity of mixed phosphate eluates of the neutral lead acetate precipitate prepared in the same way as the eluates of part (c) except that instead of the acid phosphate elutions the precipitates of (b) were treated with water. Water removes no virus from the neutral lead acetate precipitate.

Inoculations were made according to the method of Holmes (4) in which the concentration and quantity of virus inoculated are roughly regulated to give about 50 per cent of the plants diseased. Juice samples and eluates were inoculated at a 1 to 3 dilution based on the original juice. Juice samples were diluted with two volumes of water and eluates were diluted with two additional volumes of the eluate obtained by a second elution of the residue.

In Table IV, column 5, are given the odds that the difference in plants diseased between control juice and preparation represents a real difference in virus concentration. In two of the five preparations of inocula (c) there is no likelihood favoring a difference in virus concentration. In Experiment 2 the ratio 24 to 1 is barely significant.

The dilution corresponding to the difference in number of plants diseased in inoculation with preparations and control juice samples may be obtained from the dilution curve (4). The percentages corresponding to these dilutions are given in column 6. The dilution curve affords some scale for conversion of the difference in numbers of plants diseased and the difference in virus concentration. The figure in column 5 is not a factor weighting the percentage; column 5 gives the likelihood that any difference whatever exists.

In Experiments 1 and 2, the eluates obtained when potassium acid phosphate elution was omitted show somewhat less activity than those prepared in the usual way (c). The likelihood in column 7 supporting a difference in virus concentration in favor of preliminary acid phosphate elution exceeds 20 to 1, that required for significance, but is not of a high order of certainty. In Experiment 3 the difference favors the contrary case, but the likelihood is short of that required for significance.

Tobacco mosaic virus freed from 98 to 99 per cent of the organic im-

TABLE VII  
RESULTS OBTAINED ON ELUTING THE NEUTRAL LEAD ACETATE PRECIPITATE OF THE  
VIRUS WITH DILUTE ALKALI SOLUTIONS

Preparation number	500 cc. of juice from fresh (unfrozen) diseased plants, used as starting material			Neutral lead acetate precipitate of the virus eluted with $M/3$ $KH_2PO_4$ ; virus removed from the precipitate with dilute $Na_2CO_3$ in the presence of amyl alcohol			Inoculations* with starting material			Inoculations with the preparations			Likelihood of a difference
	Total solids in grams	Total nitrogen in grams		Total solids in grams	Total ash in grams	Total nitrogen in grams	Dilution	No. of plants inoculated	No. of plants diseased	Dilution	No. of plants inoculated	No. of plants diseased	
957	15.0325	0.4012		0.5050	0.1130	0.0620	1:4	100	61	1:4	100	36	10 <sup>3</sup> :1
951A	14.1250	0.4300		0.3800	0.0510	0.0600	1:1	100	49	1:1	100	42	3:1
948	13.9462	0.4602		0.2825	0.0660	0.0350	1:1	150	73	none	150	43	
950A	25.2137	0.3725		0.2100	0.0280	0.0330	none	100	31	none	100	45	15:1
944B	12.6825	0.3925		0.2825	0.0345	0.0390	none	10	7	none	10	7	
	500 cc. of juice from frozen diseased plants, after thawing, used as starting material			Juice cleared with basic lead acetate and the virus precipitated with neutral lead acetate solution. Subsequent elution and recovery of the virus from the precipitate as above									
936A	11.6987	0.4100		0.0815	0.0285	0.0080	none	10	4	none	10	4	
840	13.4887	0.3125		0.1145	0.0290	0.0105	none	10	9	none	10	10	
841	13.4887	0.3125		0.0845	0.0240	0.0075	none	10	9	none	10	8	

\* Inoculations made by five pin punctures in one leaf per plant except preparations 840 and 841 in which four leaves per plant were inoculated.



purities accompanying it in the plant juice can be prepared according to the procedure in Table VII. The preparations obtained are highly active and in two of the instances reported fully as active as the original juice. Inactivity of the M/3 acid phosphate eluate which removes pigment but no virus from the neutral lead acetate precipitate could not be demonstrated to be the result of inactivation of the virus. When this step is omitted from the procedure the slight difference observed favors acid phosphate elution.

OUTLINE OF THE PROCEDURE FOR REMOVING THE VIRUS FROM JUICE OF  
DISEASED PLANTS USING NEUTRAL LEAD ACETATE AS  
THE PRECIPITATING AGENT

500 cc. of juice from frozen diseased plants (13) plus 15 cc. of Horne's basic lead acetate solution (200 grams in 1000 cc.  $H_2O$ ), centrifuge.

Decant the supernatant liquid and add 35 cc. of a neutral lead acetate solution, prepared by dissolving 200 grams of neutral lead acetate in 1000 cc. of water. Let stand 15 minutes, centrifuge.

Discard the precipitate

Wash the cream colored precipitate once, using 500 cc. of distilled water, centrifuge.

Discard the supernatant liquid

Suspend the cream colored precipitate in 800 cc. of M/3 primary potassium ortho-phosphate solution, centrifuge.

Discard the supernatant liquid

Suspend the white precipitate in 800 cc. of M/3 solution of primary potassium ortho-phosphate, centrifuge.

Discard the colored supernatant liquid

Wash the white precipitate twice, using 500 cc. of distilled water each time, centrifuge.

Discard the colored supernatant liquid

Suspend the white precipitate in about 400 cc. of distilled water, then add 100 cc. of a mixed phos-

Discard the washings

phate solution, prepared by adding 200 cc. of M/3 primary potassium ortho-phosphate to 300 cc. of M/3 secondary potassium ortho-phosphate solution. Let the suspension stand for about two hours at room temperature, shaking frequently. Place at a temperature below freezing overnight, then thaw on the centrifuge.

#### The virus solution

#### Precipitate

The above procedure should be completed in about 24 hours and the precipitate suspended in the neutral phosphate solution as soon as possible once the preparation is started.

Since the virus is not removed from the lead precipitate at pH 4.5, but is at 6.5, it became the practice to adjust the hydrogen ion concentration of the juice to about pH 6.5 with a solution of sodium carbonate before clearing with basic lead acetate solution. After clearing, the reaction was adjusted to a hydrogen ion concentration of about pH 5.0 and the virus precipitated with neutral lead acetate solution. A M/3 solution of primary potassium ortho-phosphate has been used to adjust the hydrogen ion concentration of the cleared juice. Loss of virus observed in clearing the juice with basic lead acetate solution is avoided by adding 200 cc. of a M/3 solution of primary potassium ortho-phosphate to 500 cc. of juice from diseased plants and the virus precipitated at once with neutral lead acetate solution. The organic solid content of this precipitate, after acid phosphate elution, is higher, however, than that obtained after first clearing the juice with basic lead acetate solution.

#### REDUCED ASH CONTENT OF VIRUS PREPARATIONS OBTAINED FROM NEUTRAL LEAD ACETATE PRECIPITATE WITH DILUTE ALKALI

Elution of the lead precipitate of the virus with neutral phosphate solution is quite effective in removing the virus, but has the disadvantage of introducing relatively large amounts of inorganic salts into the solution. This, of course, is unsatisfactory when the primary object is to isolate the virus. Attempts have been made, with partial success, to elute the virus from the lead precipitate with solutions of organic bases such as pyridine and quinoline. The base was then extracted from the virus solution with ether. The virus is effectively released from the lead precipitate when a suspension is brought to pH 6.5 by adding 0.5 to 0.6 cc. of a saturated solution of sodium carbonate to 500 cc. of the suspension. In this way highly infectious solutions, containing only a few decigrams of ash in the preparation obtained from 500 cc. of juice, have been obtained (see Table VII). Amyl alcohol was observed to exert some effect in removing the virus from

the lead precipitate, hence it has been the practice to use amyl alcohol along with a dilute solution of sodium carbonate.

Table VII gives the results obtained on eluting the virus from the lead precipitate by means of a dilute solution of sodium carbonate in the presence of amyl alcohol. The inoculation experiments with such preparations, using only 10 plants in an experiment, indicate that the infective power of the preparations approximated that of the starting material. Inoculation experiments using larger numbers of plants (Table VII) indicate that at least solutions No. 950A and 951A may have equalled the starting material in infective power.

The ash content of the solutions given in Table VII can be largely accounted for by the sodium carbonate added. It is readily seen that the percentage of nitrogen on the basis of the organic matter present is about the same as in some of the simple proteins. Preliminary clearing of the juice with basic lead acetate solution drastically reduces the organic matter and nitrogen content of the neutral lead acetate precipitate. From such a precipitate, however, a mixed phosphate eluate has been obtained which shows no significant difference in infective power when compared with the starting material—both showing high infective power (see Table IV, Exps. 1 and 5).

#### ISOLATION OF ACTIVE CRYSTALLINE MATERIAL

Many attempts have been made to isolate an active crystalline product from the acetone precipitate obtained after clearing the juice with lead acetate and barium acetate solutions. These attempts were uniformly unsuccessful when purely aqueous solutions of the virus were used. Crystals obtained from purely aqueous solutions, when thoroughly rinsed, gave inactive solutions. An active crystalline product has been obtained, however, from dilute acetone solution. In order to obtain an active crystalline product acetic acid is added to the concentrated solution of the acetone precipitate to about pH 5.0. Acetone is then added slowly, with constant stirring, until a slight permanent cloud appears. The solution is then placed in the ice box where crystalline material separates on standing. Moderately active products of crystals uniform in appearance have been obtained in this way. Such products have retained much of their activity following recrystallization. About 33 per cent of the crystalline product is ash. The crystalline product, therefore, does not represent pure virus since the ash, which is largely calcium oxide, can be greatly reduced through the safranin procedure and also through the lead precipitation of the virus without losing infective power.

Crystals have been obtained from some of the more highly purified solutions described in this paper. The yield has been so slight, however, that no tests have been made.

## INCREASED ACTIVITY IN PRESENCE OF SUSPENDED MATERIAL

In discussing the precipitation of virus in the precipitate obtained on clearing with basic lead acetate, the high infectivity of suspensions obtained on washing the precipitate was considered to be abnormal and due to an effect of suspended material exaggerating the infectivity of a low concentration of virus. The demonstration of the exaggeration of the infectivity of a dilute virus sample in suspensions is herewith reported. For the following inoculations a juice sample expressed from diseased plants frozen then thawed was diluted to 20 volumes with distilled water. Suspensions were prepared by adding 2 gm. of talc in one series and 2 gm. of norite in another to 50 cc. of the diluted virus solution. The 1 to 20 virus and the two suspensions were inoculated on flatted plants by puncturing one leaf with five pins, using Holmes' method (4).

As with the inoculation reported in Table IV the percentage increase is included to afford a basis of comparison of the activity of the preparation and starting material with a sample of diseased juice on dilution. The likelihood, column 7, gives odds that any difference exists and does not weight directly the percentage difference reported.

The results discussed above and reported in Table VIII indicate that in the presence of suspensions of inactive substances the infectivity of a preparation or juice sample may be increased far beyond that corresponding to the actual concentration of virus present.

TABLE VIII  
INCREASE IN INFECTIVITY OF JUICE SAMPLE BY SUSPENDED MATERIAL

Preparation	Plants inoculated	Plants diseased	Increase in apparent virus concentration	Likelihood of a difference
1/20 virus	162	57		
1/20 virus+talc	160	96	12-fold	> 10 <sup>4</sup> :1
1/20 virus+norite	159	114	37-fold	> 10 <sup>4</sup> :1

## DISCUSSION

In the precipitation and purification of the virus by safranin precipitation, salting out, precipitation with alcohol or acetone, crystallization of active material from dilute acetone, and precipitation by lead acetate—in all these procedures the virus behaves in a manner analogous to an emulsoid colloid, but certainly not as a suspensoid.

The behavior of the virus with safranin parallels that of some of the enzymes. Safranin precipitates the virus from solution and also is used as a precipitant for the proteoclastic enzymes (10, 5, 7). The virus is freed from the safranin precipitate by the same means as that used for freeing pepsin from the safranin precipitate (7). Safranin has also been used as a precipitant for proteins.

Of the various theories, therefore, put forward as to the nature of the virus, the enzymic one advanced by Woods (15) and further emphasized by Freiberg (2) fits in with the experimental results here reported.

Duggar and Armstrong (1) found that the virus of mosaic disease of tobacco passed through a given filter to about the same extent as a 1 per cent hemoglobin solution. Assigning a diameter of  $30 \text{ m}\mu^1$  to the particles in a 1 per cent hemoglobin solution, they considered the virus to have the same diameter. Svedberg and Nichols (11) have more recently determined the molecular weight of the particles in a 1 per cent hemoglobin solution to be about 66,800. Assigning a density of 1.33 to these particles, the molecular diameter is calculated to be  $5.4 \text{ m}\mu$ . Northrop and Anson (8) have calculated, through determining the diffusion coefficient, the radius of carbon monoxide hemoglobin to be about  $2.73 \text{ m}\mu$ . From the conclusion of Duggar and Armstrong (1) that the virus particle is of the same size as the particles in a 1 per cent hemoglobin solution, it follows that the virus must have a molecular diameter in the solutions they employed, of 5 to  $6 \text{ m}\mu$ . This molecular diameter is of the same order of magnitude as that reported for the bacteriophage (3, 6), and is the same as that of the enzymes (14, p. 8).

The percentage of nitrogen in the more highly purified fractions, on the basis of the organic content, is very close to that for some of the simple proteins. The fact that in purification of the virus fraction the percentage of nitrogen increases as the total solid content is reduced argues also for its nitrogenous character.

#### SUMMARY

The safranin precipitate of the virus has not been found to yield a fraction favorable for the isolation of a product free from pigment. In all solutions of the virus obtained from the safranin precipitate brown pigment is present.

Adding hydrochloric acid to a cleared juice sample until the hydrogen ion concentration is equal to about pH 5.0, then precipitating with acetone in the cold, yields a highly infectious product with a solid content only ten per cent, or less, of that of the original juice sample.

A solution of lead acetate may be used to precipitate the virus from juice of diseased Turkish tobacco plants.

The virus is released from the lead precipitate upon suspending the precipitate in a M/15 solution of potassium phosphate of hydrogen ion concentration of about pH 6.5.

Suspending the lead precipitate of the virus in a normal solution of primary potassium ortho-phosphate does not remove an appreciable amount of the virus, but does remove irrelevant organic material. This makes elu-

<sup>1</sup>  $\text{m}\mu = 1 \text{ millimicron} = 10^{-6} \text{ mm}$ . Rivers (9, p. 11), Uhler (12).

tion of the lead precipitate with acid phosphate solution very desirable before eluting with neutral phosphate solution.

Virus solutions have been obtained from the lead precipitate which were equal in infective power to the original juice, but with a total solid content of only about one per cent of that of the original juice sample.

The basic lead acetate precipitate, obtained on preliminary clearing of the juice, removes some virus.

Crystals uniform in appearance have been obtained from the solution of the acetone precipitate. These crystals are moderately active and much of their activity is retained on recrystallization. The ash content of the crystals was high as compared with other more highly active virus preparations.

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# EFFECT OF ENZYMES UPON THE INFECTIVITY OF THE VIRUS OF TOBACCO MOSAIC

MARY LOJGIN AND CARL G. VINSON

## INTRODUCTION

As shown in the previous report by Vinson and Petre (5), preliminary experiments on the digestion of the acetone precipitate of the virus by the use of proteolytic enzymes indicated that trypsin and the combination of trypsin and pepsin inactivated the virus. The present paper gives a report of more extensive experiments with various enzyme preparations and combinations using as substrate a solution of the acetone precipitate, the neutral phosphate eluate of the lead precipitate of the virus (6), the dilute sodium carbonate eluate of the lead precipitate of the virus (6), and the untreated juice expressed from diseased Turkish tobacco plants, using that from both frozen and fresh plants. The enzyme preparations used were emulsin, pepsin, yeast extract, erepsin, papain, pancreatin, and trypsin. The enzyme preparations were obtained from a commercial source and their activity was checked on suitable substrates.

## PROCEDURE

Fifty milligrams of the enzyme preparation were suspended in 25 cc. of the virus preparation and the mixture incubated for various periods at about 37.5° C. Chloroform and toluene were used as preservatives and all samples were maintained in a state free from bacterial and fungous growth. As a control a sample of the virus solution was also incubated at the same time without the addition of enzyme. At the end of 48 hours, 72 hours, and longer periods, the digests and control were removed from the incubator, mixed, and samples for inoculation were pipetted out. Mixing was necessary before taking the sample since some precipitate invariably formed on incubation. In order to determine whether the virus was in the supernatant liquid or in the precipitate, the sample after incubation was in some instances centrifuged, the supernatant liquid decanted, and the precipitate washed three times and suspended in distilled water. Inoculations were then made with the original supernatant liquid and also with the suspension of the precipitate.

In each experiment 10 plants were inoculated by puncturing 4 leaves of each plant with 5 pins, making a total of 20 punctures per plant. Each experiment was repeated until consistent results were obtained. Unless otherwise stated, the plants used for inoculation were *Nicotiana tabacum* L. variety Turkish; but in order to supplement the results obtained with Turkish tobacco plants, some experiments were also carried out with *Nicotiana glutinosa* L. In this case the inoculation consisted of rubbing the



surface of the leaves with a piece of cotton dipped into the suspension under investigation, and then washing the excess off the leaves with water. The number of lesions formed on the inoculated leaves served to indicate the degree of infectivity of the solution used, as shown by Holmes (2, p. 47-53).

### RESULTS

The results with the emulsin preparation are shown in Table I. Columns 5 and 6 in Table I, giving the number of diseased plants resulting

TABLE I  
EFFECT OF EMULSIN UPON THE INFECTIVITY OF THE VIRUS

Virus preparation	pH	Lot No.	Incubation period, days	Number of plants diseased out of 10 inoculated	
				With the digest	With the control
A solution of the acetone precipitate	4.7	77	2 3	4 8	9 8
		90	2 3	9 9	9 10
		72	2 3	10 9	10 9
Dilute sodium carbonate eluate of the lead precipitate	6.4	86	2 3	6 5	7 6
	7.0	116	2 3	6 6	7 6
Neutral phosphate eluate of the lead precipitate	6.9	219	2 3 7 14 21	10 10 9 10 10	10 9 9 10 10
Juice from fresh diseased tobacco plants		96	2 3	10 9	8 8
		108	2 3	9 9	9 10

from the inoculations with the digest liquid as compared with the untreated virus preparation, show that the emulsin was without appreciable action on the virus in any of the solutions used. The same preparation was active, however, in hydrolyzing the  $\beta$ -glucosides, salicin, and amygdalin.

The pepsin solution when added alone also failed to reduce materially the infectivity of the virus; these tests were similar to those shown in Table I; about 220 plants were included in the pepsin tests in 11 experiments; in some cases the period of digestion was extended to more than 20 days, in one case to 28 days. This lack of action may have been due to an unfavor-

able hydrogen ion concentration, since the virus, without the addition of enzyme, is inactivated to a considerable extent at the optimum reaction for pepsin. In one experiment the virus solution was brought to a hydrogen ion concentration of pH 3.3, and the pepsin preparation added. At this reaction inactivation of the check also took place.

The yeast extract likewise was without pronounced effect in lowering the infectivity of the virus preparation. This conclusion is based on 10 experiments using 200 plants, varying the period of digestion from 48 hours to 28 days, and testing the solution of the acetone precipitate, the dilute sodium carbonate eluate, and the neutral phosphate eluate of the lead precipitate. However, as shown in Table VIII, lot No. 264, when inoculations were made with *Nicotiana glutinosa* L. digestion of the neutral phosphate eluate with yeast extract decreased significantly the capacity to produce the lesions characteristic of the disease on this species.

The erepsin preparation gave only slight evidence of inactivating the virus when the digestion was carried out over the 48 and 72 hour periods (see Table II). In some experiments (Table II), however, the incubation was extended over a period of several days and these tests showed that the infectivity of these digests underwent a progressive decrease, while the control held up in infective power. These experiments are significant since they recall the action of erepsin on another substance — glutathione. It has been noted (3) that the action of erepsin on glutathione is evident only after an incubation period of eight to ten days. In lot No. 224 of Table II, however, the check also lost activity.

TABLE II  
EFFECT OF EREPSIN UPON THE INFECTIVITY OF THE VIRUS

Virus preparation	pH	Lot No.	Incubation period, days	Number of plants diseased out of 10 inoculated	
				With the digest	With the control
A solution of the acetone precipitate		20	2	9	6
			3	6	9
		33	2	10	9
			3	8	8
		37	2	8	10
			3	8	10
		66	2	10	10
			3	8	6
Dilute sodium carbonate eluate of the lead precipitate	6.4	83	2	4	7
			3	5	6
	7.0	113	2	4	7
			3	4	6

TABLE II (continued)  
EFFECT OF EREPSIN UPON THE INFECTIVITY OF THE VIRUS

Virus preparation	pH	Lot No.	Incubation period, days	Number of plants diseased out of 10 inoculated	
				With the cigest	With the control
Neutral phosphate eluate of the lead precipitate	6.8	191	2	9	10
			3	7	9
			8	6	10
			12	5	10
			16	3	10
	7.6	224	2	8	10
			3	4	9
			7	5	9
			14	5	2
			21	3	4
	7.1	252	28	2	1
			18	7	10
			24	6	8
			27	3	10
	8.0	268	40	2	9
			5	1	8
	7.8	244*	7	1	8
			21	1	10
			27	1	7
			29	1	7
			32	0	9
	7.7	284	37	1	9
			4		6
			4	5	9†
			13		10
			13	5	9†
			21		8
			21	2	7†
	7.7	288	28		7
			28	2	5†
			4		7
			4	4	7†
			13		8
			13	2	6†
	7.7	298	21		5
			21	0	7†
			28		4
			28	0	3†
Juice from fresh diseased tobacco plants	7.2	154	7		10
			7	4	6†
	5.6	163	2	8	9
			3	9	10
			7	9	10
			8	10	9

\* 50 mgs. of erepsin added every 7 days.

† Incubated with inactivated erepsin.

TABLE III  
EFFECT OF PAPAIN UPON THE INFECTIVITY OF THE VIRUS

Virus preparation	pH	Lot No.	Incubation period, days	Number of plants diseased out of 10 inoculated			
				With the digest	With the control		
A solution of the acetone precipitate		54	2 3	10 10	10 10		
		68	2 3	5 5	10 6		
Dilute sodium carbonate eluate of the lead precipitate	6.4	85	2 3	1 2	7 6		
	7.0	115	2 3	0 0	7 6		
	7.0	122	2 3	5 5	9 10		
	7.0	137	2 3	0 0	9 4		
Neutral phosphate eluate of the lead precipitate	6.8	190	2 3 8 12 16	1 0 1 0 0	10 9 10 10 9		
			2 3 9 17	0 0 0 0	8 8 9 9		
			6.8	207	2	27*	100**
			7.1	249†	1 hr.† 2 " 4 " 5 " 7 " 30 "	7 5 3 3 3 1	10 10 10 10 10 9
			7.1	257†	1 hr.† 2 " 3 " 4 " 5 " 6 " 24 "	4 3 6 6 6 3 3	10 10 10 10 10 10 10
	6.8	261	2	1	9		
	5.0	272	2	1	10		
	5.0	272	4	0	9		
	Juice from fresh diseased tobacco plants		97	2 3	9 8	8 8	
			160	2 3	10 8	9 10	

\* Out of 161 plants inoculated; \*\* Out of 159 plants inoculated.

† Note that in lot Nos. 249 and 257 the incubation periods are given in hours instead of days.

TABLE IV  
DIGESTION OF A SOLUTION OF THE ACETONE PRECIPITATE OF THE VIRUS  
WITH PROTEOLYTIC ENZYMES

Enzyme	Lot No.	Inoculated with precipitate or with supernatant liquid	Incubation period, days	Number of plants diseased out of 10 inoculated	
				With the digest	With the control
Trypsin	57	liquid	2 3	4 7	10 10
		precipitate	2 3	6 5	10 10
	64	liquid	2 3	8 3	10 6
		precipitate	2 3	3 5	10 6
Trypsin and erepsin	65	liquid	2 3	4 3	10 6
		precipitate	2 3	4 4	10 6
Trypsin, pepsin and erepsin	59	liquid	2 3	2 3	10 10
		precipitate	2 3	7 7	10 10
Pepsin	56	liquid	2 3	10 10	10 10
		precipitate	2 3	9 5	10 10
Papain	71	liquid	2 3	1 2	10 9
		precipitate	2 3	6 6	10 9
	91	liquid	2 3	4 3	9 10
		precipitate	2 3	4 3	9 10
Erepsin	58	liquid	2 3	8 8	10 10
		precipitate	2 3	8 5	10 10

The papain preparation (Table III) exerted its most powerful effect on the virus in the neutral phosphate eluate. The action of papain on fibrin has been found to be greatly promoted by the presence of phosphate (4).

TABLE V  
INCUBATION OF VIRUS PREPARATIONS WITH COMBINATIONS OF  
PROTEOLYTIC ENZYMES

Virus preparation	Enzymes used	Lot No.	Incubation period, days	Number of plants diseased out of 10 inoculated	
				With the digest	With the control
A solution of the acetone precipitate	Trypsin and pepsin	18	2 3	2 4	7 9
		25	2 3	5 5	9 8
		30	2 3	2 4	10 8
		38	2 3	2 4	10 10
	Trypsin, pepsin and erepsin	46	2 3	2 4	10 10
		50	2 3	8 5	10 10
	Trypsin and erepsin	21	2 3	0 1	6 9
		40	2 3	4 2	10 10
		44	2 3	4 5	10 10
		52	2 3	5 5	10 10
	Erepsin and pepsin	22	2 3	9 9	6 9
		39	2 3	8 7	10 10
Dilute sodium carbonate eluate of the lead precipitate	Trypsin and erepsin	88	2 3	0 0	7 6
		118	2 3	1 1	7 6
Neutral phosphate eluate of the lead precipitate	Erepsin and pepsin	253	18	8	10
			24	9	8
			27	5	10

The appearance of the papain digest changed considerably after a few hours of incubation. A precipitate settled out of the solution leaving the supernatant liquid perfectly clear with none of the former opalescence. Before taking a sample with which to inoculate plants, the solution was

TABLE VI

EFFECT OF TRYPSIN UPON THE INFECTIVITY OF THE VIRUS

Virus preparation	pH	Lot No.	Incubation period, days	Number of plants diseased out of 10 inoculated	
				With the digest	With the control
A solution of the acetone precipitate		17	2 3	2 3	7 9
		24	2 3	2 5	9 8
		32	2 3	2 4	10 8
		36	2 3	4 3	10 10
		120	2 3	3 2	9 10
		93†	2 3	4 1	9 10
Dilute sodium carbonate eluate of the lead precipitate	6.4	82	2 3	0 0	7 6
	7.0	112	2 3	1 0	7 6
	7.0	135	2 3	2 0	9 4
Neutral phosphate eluate of the lead precipitate	6.8	188	2 3 8 12 16	0 1 0 1 1	10 9 10 10 9
	6.8	206	2	12*	100**
	6.8	196	2 3 9 17	0 0 2 2	8 8 9 10
	6.8	214	2 3 8	0 0 0	7 10 7
	7.1	248†	1 hr.† 2 " 4 " 5 " 7 " 30 "	10 4 3 2 4 2	10 10 10 10 10 9

\* Out of 162 plants inoculated; \*\* Out of 159 plants inoculated.

† Note that in lot nos. 248 and 256 the incubation periods are given in hours instead of days.

‡ 150 mgs. of trypsin added to 25 cc. of solution.

TABLE VI (continued)  
EFFECT OF TRYPSIN UPON THE INFECTIVITY OF THE VIRUS (CONT.)

Virus preparation	pH	Lot No.	Incubation period, days	Number of plants diseased out of 10 inoculated	
				With the digest	With the control
Neutral phosphate eluate of the lead precipitate (continued)	7.1	256†	1 hr. †	6	10
			2 "	9	10
			3 "	8	10
			4 "	7	10
			5 "	5	10
			6 "	8	10
			24 "	1	10
	6.8	259	2	2	9
	7.9	266	2	0	9
			5	2	8
	7.7	286	14	2	10
			22	2	10
	7.7	291	4	1	7
			14	0	8
			22	2	5
Juice from frozen diseased tobacco plants		62	2	8	9
			3	7	6
Juice from fresh diseased tobacco plants		98	2	9	8
			3	8	8
		107	2	6	9
			3	7	10
	5.6	157	2	8	9
			3	7	9
	5.6	162	7	10	9
			8	10	9
	7.2	151	2	10	9
			3	10	10
	7.8	179	2	8	9
			3	8	8
			5	6	8
			7	7	10
			9	5	8
			10	7	6
	5.6	156†	2	7	9
			3	9	9
	7.2	153†	2	7	9
			3	7	9

usually mixed so that both the precipitate and the supernatant liquid were inoculated into the plant together. In order to determine which fraction



TABLE VII  
EFFECT OF PANCREATIN UPON THE INFECTIVITY OF THE VIRUS

Virus preparation	pH	Lot No.	Incubation period, days	Number of plants diseased out of 10 inoculated	
				With the digest	With the control
A solution of the acetone precipitate		67	2 3	2 5	10 6
		73	2 3	5 4	10 9
		78	2 3	5 1	9 8
		92	2 3	8* 3	9 10
Dilute sodium carbonate eluate of the lead precipitate	6.4	84	2 3	1 1	7 6
	7.0	114	2 3	2 2	7 6
	7.0	121	2 3	4 3	9 10
	7.0	136	2 3	0 0	9 4
Neutral phosphate eluate of the lead precipitate	6.8	189	2 3 8 12 16	0 1 0 0 0	10 9 10 10 9
Juice from fresh diseased tobacco plants	7.2	152	2 3	9 8	9 10
	5.6	159	2 3	9 10	9 10

\* Contamination.

contained the virus, some of the digests were centrifuged at the end of the incubation period. The supernatant liquid was decanted, the precipitate was washed three times then suspended in distilled water. One set of plants was inoculated with the supernatant liquid and another set of plants was inoculated with the suspension of the precipitate. A comparison of the activity of the precipitate and the supernatant liquid is shown in Table IV. It is seen that there was very little difference in the infectivity of these two fractions.

On using combinations of the enzymes on the purified virus preparation, it was found that when trypsin was present in the combination con-

siderable loss of activity took place (Table V). It was also found that trypsin alone (Table VI) apparently exerted a greater inactivating power than when combined with other enzymes. This latter result may be due to the reciprocal action of proteoclastic enzymes, since it has been reported by Effront (1, p. 498-500), that pepsin could be digested by trypsin, while erepsin tends to inactivate trypsin. A combination of pepsin and erepsin had little effect on the virus (Table V).

More nearly complete inactivation of the virus by trypsin took place in the neutral phosphate eluate than in the solution of the acetone precipitate (Table VI). This might be due to the presence of inhibitory substances in the acetone precipitate, since this preparation is not as highly purified as the neutral phosphate eluate of the lead precipitate of the virus. Strength is given to this possibility by the fact that trypsin has practically no action on the virus in juice expressed from diseased plants in either the fresh or frozen condition (Table VI). In this connection, it is interesting to note that the proteoclastic activity of juice from diseased tobacco plants is greatly reduced in the acetone precipitate, and the neutral phosphate eluate of the lead precipitate of the virus has practically no proteoclastic activity. This may explain, in part, why the virus in the neutral phosphate eluate is more susceptible to attack by enzymes than that in any other virus preparation employed.

The results with pancreatin (Table VII) show that the preparation was only slightly less powerful in inactivating the virus than the trypsin preparation.

In some of the experiments reported in Tables V and VI, the number of plants diseased which had been inoculated with the digest at the end of 72 hours of incubation exceeded the number of diseased when the plants were inoculated at the end of 48 hours of incubation. To demonstrate whether the inactivation observed on incubation with a trypsin preparation was permanent or temporary, and also to determine whether complete inactivation could be obtained, experiments involving long incubation periods were conducted. As shown in Tables II, VI, and VII, no noticeable increase in the infectivity of the digest was observed after prolonged incubation. There seems to be no tendency of the virus, in the trypsin digest, to recover activity even after a period of 17 days. The equilibrium in the trypsin digest is evidently attained at the end of about 48 hours.

Table VIII gives the results obtained by digesting the neutral phosphate eluate with various enzymes and inoculating plants of *Nicotiana glutinosa* L. with the various digests. As seen from this table, both trypsin and papain gave evidence of inactivating the virus. Considerable reduction of activity was produced by erepsin when the hydrogen ion concentration of the virus preparation was brought close to the optimum pH for erepsin. This inactivation by erepsin was more nearly complete when

TABLE VIII  
EFFECT OF ENZYMES UPON THE CAPACITY OF THE NEUTRAL PHOSPHATE ELUATE  
TO PRODUCE LESIONS IN LEAVES OF NICOTIANA GLUTINOSA L.

Enzyme added	pH	Lot No.	Incubation period, days	Inoculated with the digest		Inoculated with the control	
				No. leaves inoculated per plant	No. lesions per leaf	No. leaves inoculated per plant	No. lesions per leaf
Trypsin	6.8	259	2	8	0.3	8	27.0
"	7.9	266	2	8	0.3	8	17.0
"	7.9	"	3	8	0.0	8	40.0
"	7.9	"	6	8	0.1	8	13.0
"	7.9	"	12	10	0.5	10	3.2
Papain	6.8	261	2	8	0.3	8	27.0
"	5.0	272	2	8	1.3	8	53.0
"	5.0	"	5	8	0.9	8	18.0
"	5.0	"	11	10	0.2	10	20.0
Erepsin	6.8	263	2	8	18.5	8	27.0
"	8.0	268	2	8	3.0	8	17.0
"	8.0	"	3	8	2.3	8	40.0
"	8.0	"	6	8	0.5	8	13.0
"	8.0	"	12	10	0.4	10	3.2
"	7.7	284	11	12	0.9	12	13.0
"	7.7	"	11	—	—	12	15.0*
"	7.7	"	33	7	0.7	7	10.0
"	7.7	"	33	—	—	6	30.5*
"	7.7	"	36	6	0.8	6	6.6
"	7.7	288	11	12	0.1	12	3.6
"	7.7	"	11	—	—	12	5.0*
"	7.7	"	33	8	0.2	9	5.0*
"	7.7	"	36	6	0.0	6	5.2*
"	7.7	298	7	7	3.2	7	6.3
"	7.7	"	7	—	—	8	5.6*
Pancreatin	6.8	260	2	8	4.5	8	27.0
Pepsin	6.8	262	2	8	8.7	8	27.0
Yeast	6.8	264	2	8	3.0	8	27.0
Emulsin	6.8	265	2	8	12.0	8	27.0
Pepsin	3.5	269	2	8	28.5	8	31.8
"	3.5	"	5	8	14.8	8	17.1
"	3.5	"	11	10	6.1	10	12.1

\* Incubated with inactivated erepsin.

the incubation was extended over longer periods. Pancreatin and yeast extract also exerted some inactivating effect on the virus, but pepsin and emulsin showed no decided tendency to inactivate the virus in the preparations used.

None of the enzyme preparations used was effective in inactivating the virus in untreated juice, either from frozen or fresh plants.

#### EVIDENCE THAT INACTIVATION OF THE VIRUS WAS DUE TO THE ACTIVITY OF THE ENZYME PREPARATION ADDED

In order to determine whether reduction of infectivity of a virus preparation was due to the activity of trypsin or to substances accompanying the trypsin, the experiments recorded in Table IX were carried out.

TABLE IX

RESULTS OF INCUBATING THE VIRUS PREPARATION WITH INACTIVE ENZYMES AND OF TREATING THE VIRUS WITH ENZYMES IN THE COLD

Virus preparation	Enzyme added	Lot No.	Temp. of incubation °C.	Period of incubation	Number of plants diseased out of 10 inoculated	
					With the digest	With the control
A solution of the acetone precipitate	Inactive trypsin and inactive pepsin	26	37.5	48 hrs. 72 "	8 8	9 8
	Inactive trypsin and inactive erepsin	45	37.5	48 " 72 "	10 9	10 10
	Inactive trypsin+ *pepsin and *erepsin	47	37.5	48 " 72 "	10 10	10 10
	Inactive trypsin+ *pepsin and *erepsin	51	37.5	48 " 72 "	10 9	10 10
	Inactive trypsin and *erepsin	53	37.5	48 " 72 "	9 10	10 10
	Trypsin	100	28	5 min.	8	10
	"	124	0	30 "	4	6
	"	140	0	30 "	7	9
	Pancreatin	141	0	30 "	4	9
	Papain	142	0	30 "	10	9
	Emulsin	143	0	30 "	10	9
Neutral phosphate eluate of the lead precipitate of the virus	Yeast extract	144	0	30 "	10	9
	Trypsin and erepsin	145	0	30 "	8	9
	Trypsin	228	0	30 "	8	9
	Pancreatin	229	0	30 "	6	9
	Papain	230	0	30 "	5	9
	Trypsin	232	0	30 "	6	10
	Pancreatin	233	0	30 "	9	10
	Papain	234	0	30 "	9	10
	Trypsin	238	0	30 "	7	8
	Pancreatin	239	0	30 "	6	8
	Papain	242	0	30 "	3	8

\* Inactive.

In the first set of these experiments, a suspension of the enzyme preparation was inactivated by boiling. The suspension was then cooled and an aliquot added to a virus sample and incubated in the usual way. A check correspondingly diluted with distilled water was prepared at the same time and likewise incubated. Ten sets of experiments conducted with inactive trypsin and combinations of inactive trypsin with inactive pepsin and inactive erepsin gave an average of 9.3 plants diseased out of every 10 plants inoculated. The checks gave an average of 9.7 plants diseased out of every 10 plants inoculated.

In the second set of experiments, an active enzyme preparation was added to a virus solution which had been cooled to almost 0° C. The digest was held at this temperature for 30 minutes and then surrounded by ice during the process of inoculating plants in the greenhouse. An average of 6.5 plants became diseased out of every 10 plants inoculated with the digest which contained trypsin, while an average of 8.5 plants became diseased out of every 10 plants inoculated with the check. This indicates that inactivation of the virus by trypsin preparations is not due to adsorption; since in this set of experiments conditions were made favorable for adsorption, but unfavorable for hydrolytic activity by the trypsin.

In order to demonstrate whether the inactivation of the virus by trypsin is due to actual digestion or to the presence of the digestion products of trypsin which may have an inhibitory effect on the virus, the following experiments were carried out: 1 cc. of virus preparation was added to 5 cc. of trypsin digest (Table X); this solution was inoculated, without incu-

TABLE X  
EFFECT OF TRYPSIN DIGEST UPON THE INFECTIVITY OF THE  
VIRUS (INOCULATION WITHOUT INCUBATION)

Results with <i>Nicotiana tabacum</i> L. variety Turkish			Results with <i>Nicotiana glutinosa</i> L.				
Lot No.	No. of plants diseased out of 10 inoculated		Lot No.	Inoculated with the digest + virus		Inoculated with the control	
	With the digest + virus	With the control		No. of leaves	No. of lesions per leaf	No. of leaves	No. of lesions per leaf
294	4	7	275	5	6.0	5	7.0
301	8	3	309	7	16.1	7	16.7
305	8	7	311	7	13.0	6	11.8
			313	6	12.8	6	20.2
			315	7	12.0	5	3.8

bation. A solution consisting of 1 cc. of virus preparation to 5 cc. of water was used as a control. The average number of lesions per leaf, obtained as a result of five experiments with *Nicotiana glutinosa* L., was 12.0 for the treated and 11.9 for the control. Three experiments with Turkish tobacco gave the respective averages of 6.7 and 5.7 plants diseased out of each 10 plants inoculated with the same solutions. Apparently the digestion products of trypsin have no inhibitory effect on the virus.

#### DISCUSSION

Since the results in Table IX show that inactivation of the virus by enzymes is not due to adsorption, it seems likely that the inactivating effect of the enzyme solution was due to its hydrolytic effect.

Under the conditions employed pepsin did not attack the virus, while trypsin did. The papain preparation, however, was quite active in reducing infectivity of the more highly purified solutions. Hydrocyanic acid was not added to the papain preparation, and the presence of hydrocyanic acid is said to be necessary to give papain power to decompose the lower protein degradation products, the peptones, and the polypeptides (7). Tests for cyanide in the papain preparation were negative. Papain (not activated), however, has been reported to show tryptic action on fibrin (4). A few tests showed that the papain preparation used in these experiments was very active in dissolving coagulated egg albumin. The same preparation, however, when incubated with a 2 per cent bacto-peptone solution and also with a 2 per cent Witte's peptone solution, produced no increase in amino nitrogen.

Erepsin seems to act on the virus upon prolonging the digestion period. This is especially interesting since erepsin, as already noted, seems to act on the peptide glutathione only at the end of eight or ten days. Since erepsin is supposed to act only on the simple protein decomposition products, and since the erepsin preparation employed gave no increase in amino nitrogen when incubated with a solution of egg albumin, this may indicate that the virus is less complex than the simple protein.

The evidence from these experiments is somewhat conflicting as to the complexity of the virus. The papain preparation which was very effective in inactivating the virus was without effect on bacto-peptone or Witte's peptone. This would indicate that the virus is of the order of a simple protein. The fact, however, that the pepsin preparation was without effect on the virus preparations at the hydrogen ion concentrations employed, whereas both trypsin and erepsin inactivated the virus, may indicate that the virus is less complex than the simple proteins.

#### SUMMARY

Under the conditions of the experiment, incubating the virus preparations with emulsin, pepsin, or yeast extract did not reduce the infectivity.

The trypsin preparation showed marked inactivating effect on the virus in all purified preparations. This effect was at least as strong when the trypsin was used alone as when used in combination with other enzymes. Pancreatin was only slightly less effective than the trypsin preparation.

Papain was especially effective in inactivating the virus in neutral phosphate solution.

The erepsin preparation was effective in reducing the infectivity of the virus only after an incubation period of several days.

None of the enzyme preparations used was effective in inactivating the virus in untreated juice, from either frozen or fresh plants.

The capacity of the enzyme solutions to reduce the infectivity of the virus preparations was destroyed by boiling.

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# LOCAL LESIONS OF MOSAIC IN NICOTIANA TABACUM L.

FRANCIS O. HOLMES

## INTRODUCTION

Necrotic local lesions, resulting from the multiplication of the virus of tobacco mosaic in the leaves of five species of *Nicotiana* L., were described in a previous paper (2). These five species were *N. glutinosa* L., *N. rustica* L., *N. acuminata* Hook., *N. langsdorffii* Schrank, and *N. sanderae* Hort. Sander. Subsequent work has shown that necrotic local lesions are formed by this virus in several other solanaceous hosts: *N. alata* Link and Otto, *N. glutinosa* L.  $\times$  *glauca* R. Grah., *Datura stramonium* L., *Solanum pseudocapsicum* L., *S. melongena* L. (commercial variety Black Beauty), and *Capsicum annuum* L. (commercial variety Anaheim Chili). Photographs of the necrotic lesions on leaves of these hosts are included in this paper (see Fig. 3). Price (4) produced similar necrotic local lesions on leaves of 16 varieties of *Phaseolus vulgaris* L. by inoculating with tobacco mosaic virus. The list of species and varieties of plants which are known to respond to inoculation with the virus of tobacco mosaic by the production of necrotic local lesions has thus been considerably lengthened.

No necrotic local lesions appear on inoculated leaves of *N. tabacum* L., and it has been commonly supposed that no local effects of any kind are produced in this plant as a result of inoculation with typical tobacco mosaic virus. During the course of the present study, however, local symptoms have been detected in *N. tabacum*, variety Turkish (Turkish tobacco), and have been made more conspicuous by applying iodine as a starch stain. The affected areas, which constitute local lesions of a mild type, do not become necrotic, but show their diseased condition by abnormal color and starch content. A very brief description of the first observation of these local lesions was published in 1929 (2, p. 40) in connection with the description of necrotic local lesions produced by this virus in other hosts. Further study has shown that the lesions may prove useful when it is desired to demonstrate the positions of points of entrance of virus, or to determine their approximate number. They may also serve to throw some light on the behavior of the virus upon entering the plant tissues.

The purpose of this paper is to describe the local lesions in *N. tabacum*, to show photographs of them, and to give evidence that the lesions seen in the living leaf coincide with areas in which virus has reached a high concentration, and in which the distribution of starch has been disturbed.

## LESIONS IN THE LIVING LEAF

It was found that pricking a green leaf of Turkish tobacco with a fine needle or insect pin previously dipped in an extract of a mosaic plant pro-



duced small circular wounds, some of which were surrounded after three or four days by pale yellowish-green halos. The halos marked the locations of local lesions, the characteristics of which are described in the following sections of this paper. Pricking a leaf with a needle not contaminated with virus produced wounds all of which healed quickly, leaving the surrounding leaf tissues unchanged in color.

The results of rubbing with a cloth were similar with respect to the formation of yellowish lesions. Light rubbing with a cloth moistened with juice from a mosaic plant resulted after some days in the appearance of numerous, often inconspicuous, yellowish spots similar to those surrounding the infective pin pricks previously mentioned. Light rubbing with a cloth wet with water, but not contaminated with juice from mosaic plants, did not affect the uniformity of the green coloration of the leaf.

Such methods of inoculation as crushing or repeatedly scratching leaves were not used, because in preliminary experiments it was found that they often caused disturbances which overshadowed those associated with the local accumulation of virus.

The local lesions did not always appear on leaves which had been infected by inoculation. They seemed to be sensitive to changes in growing conditions. It was difficult and sometimes impossible to see them in late autumn, winter, and early spring, but from April to September during the past three years the conditions in the greenhouses of the Boyce Thompson Institute proved favorable for their development.

#### LESIONS AFTER STAINING LEAF WITH IODINE

Leaves showing yellowish local lesions were left overnight in 95 per cent ethyl alcohol and then stained in a solution of iodine in potassium iodide (iodine 10 grams, potassium iodide 30 grams, water 1500 cubic centimeters). Before examination the leaves were washed with 30 per cent ethyl alcohol to remove excess iodine. The stained leaves displayed patterns corresponding to the indistinct yellowish lesions in the green leaf, showing that the distribution of starch in the leaf tissue had been modified by the presence of the virus. Starch patterns were detected even in seasons when the yellowish lesions were invisible, as in late autumn, winter, and early spring.

An inoculated leaf is shown in Figure 1 A as it appeared in the living condition, and in Figure 1 B as it appeared after it had been stained with iodine. In the living leaf one of the inoculating pin punctures was surrounded by a pale yellowish-green area. The same lesion was visible after the leaf was stained with iodine, but the contrast in color was more conspicuous in the stained preparation than it had been in the living leaf. In iodine the lesion appeared as a gray area on a yellowish-brown background. Comparison of the two photographs of the leaf will show the identity of the

position of the yellowish area on the living leaf and of the gray area after the same leaf was stained. A leaf inoculated by rubbing one-half its area with juice of mosaic plants is shown in Figure 1 C. The uninoculated half

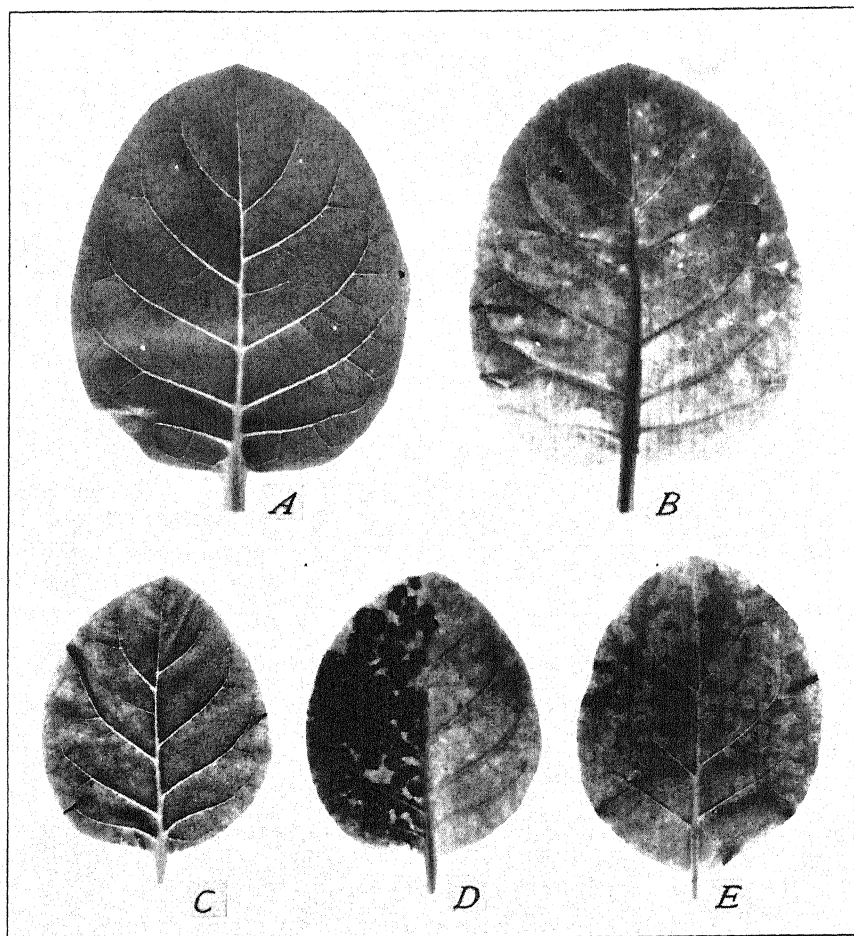


FIGURE 1. (A) Leaf of *N. tabacum* variety Turkish showing a yellowish local lesion around one of four inoculation punctures; (B) same leaf stained with iodine, showing lesion darker than background; (C) similar leaf inoculated by rubbing left half with mosaic juice; (D) same stained in iodine; and (E) leaf stained with iodine, showing lesions lighter than normal tissue.

was a uniform green in color; the green color of the inoculated half was broken up by the presence of many yellowish lesions. The same leaf stained with iodine is shown in Figure 1 D; the numerous lesions were dark gray in

color, and contrasted sharply with the yellowish-brown background of normal tissue.

Some lesions contained less and some more starch than the normal tissues. Leaves collected late in the day in summer usually showed lesions which were relatively free of starch. The patterns on these leaves appeared to be the result of a partial inhibition of photosynthesis in the infected area. An example is shown in Figure 1 E. Leaves collected early in the day in any season commonly showed lesions which contained more starch than the normal tissue. In the greenhouse, however, the rate of removal of starch was not constant. Incubation at definite temperatures in dark rooms proved more satisfactory for demonstrating lesions of this type. Such lesions were found with regularity in leaves of young plants grown in moderate sunlight, moved from the greenhouse at five o'clock in the afternoon to a dark room at 10° C., moved from this cool dark room to a dark compartment at 22° C. at nine o'clock the following morning, and held there until five o'clock in the afternoon. The lesions obtained in this way appeared to have retained much of their starch, and contrasted sharply with surrounding normal tissues which were nearly free of it. The lesions did not appear to be larger after this treatment than before. Because of their failure to increase in size during the 24-hour treatment, and because it was found that the virus did not increase appreciably in concentration when incubated at 10° C., such lesions are referred to in this paper as being of the age at which the cold treatment was begun. This type of lesion appeared to be the result of partial inhibition of translocation of starch from the infected areas. An example of this type is shown in Figure 1 D. Both of the processes by which the patterns were formed, one apparently consisting in failure to form starch at a normal rate, the other in failure to translocate starch normally, may have contributed to the formation of certain patterns in which the peripheries of the lesions appeared dark, and the interiors appeared lighter.

Isolated lesions resulting from pin puncture inoculations are shown in Figure 2. The size of lesion represented in Figure 2 A was attained three days after inoculation, and that in Figure 2 B four days after inoculation. On the fifth day after inoculation it was common to find lesions irregular in contour and extended along large veins, as in Figure 2 C, less often along small veins, as in Figure 2 D. The patterns extended along the midvein farther toward the base than toward the apex of the leaf. Nevertheless a slow extension of the pattern toward the apex along the midvein, and toward the periphery of the leaf along smaller veins, was regularly observed, as shown in a leaf 7 days after inoculation (Fig. 2 E), and in a leaf 10 days after inoculation (Fig. 2 F). Somewhat similar starch patterns have recently been described in a note by Bolas and Bewley (1) as occurring in tomato infected with aucuba or yellow mosaic. About the fifth day after in-

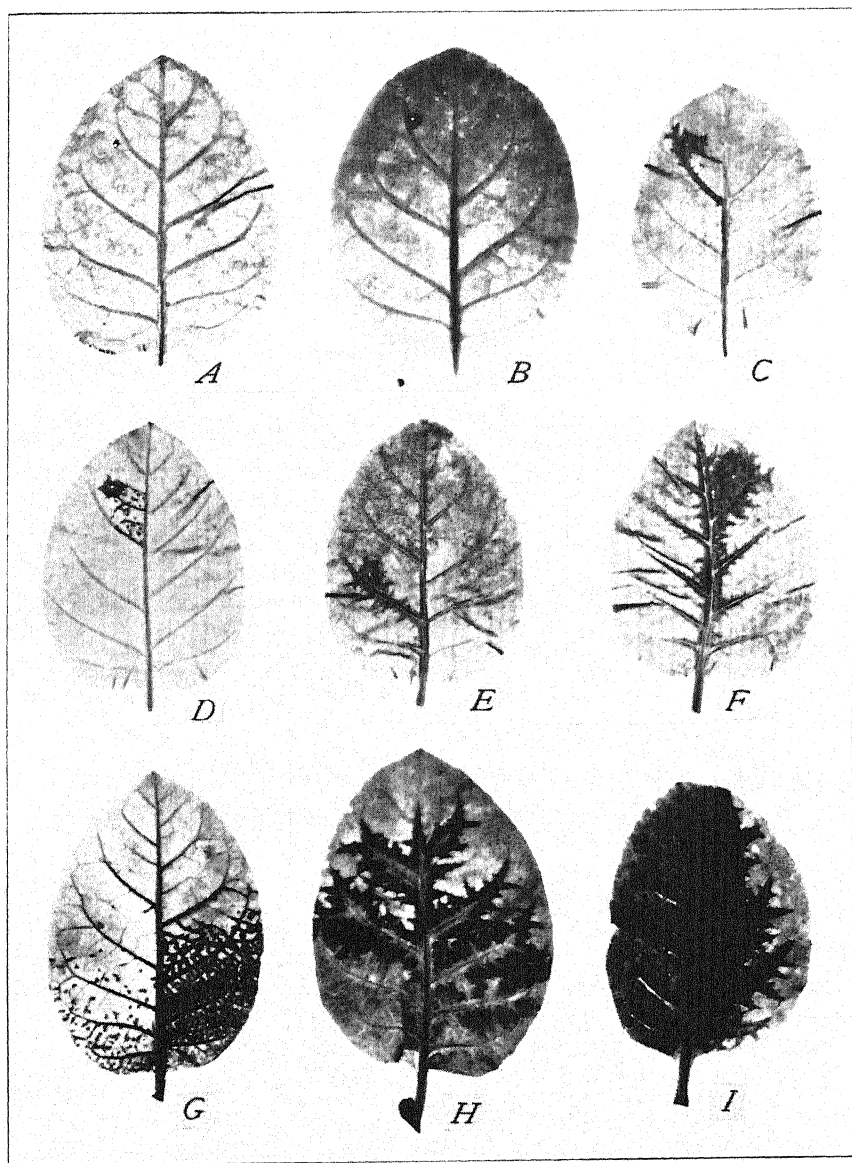


FIGURE 2. Leaves of *N. tabacum* variety Turkish showing local lesions of tobacco mosaic after iodine treatment (A) three days after inoculation, (B) four days after inoculation, (C and D) five days after inoculation, (E) seven days after inoculation, (F) ten days after inoculation. Lesions of the systemic disease on leaves showing vein-clearing (G) six days and (H) ten days after inoculation of a lower leaf of plant. (I) Leaf ten days after inoculation of left half by rubbing with extract of mosaic plants.

oculation clearing of veins occurred in the young leaves of the inoculated plants. Such leaves, when treated with iodine, showed the beginnings of secondary infections in the young tissue in which the vein-clearing had occurred. It is not the purpose of this paper to discuss the systemic phases of the disease, but pictures of this first stage of the systemic infection are included because of the occasional similarity between circular spots on some of the small veins involved in this vein-clearing pattern and those described at the site of inoculation. A leaf representing an early stage of vein-clearing, six days after inoculation of a lower leaf of the plant, is shown in Figure 2 G. In this leaf the pattern consisted of a series of dark lines bordering the veins in the young tissues near the base of the leaf, and of scattered spots, mostly elongated but occasionally circular, located on small veins. The heavier pattern on one side of the midvein is typical of this early stage of the systemic infection. In similar leaves collected later the patterns were indistinct except in the line of demarcation between long-invaded tissues at the base and still apparently normal tissues at the apex; this appearance is shown in Figure 2 H in a leaf collected 10 days after the inoculation of a lower leaf of the plant. The line of demarcation followed the veins in such a way as to resemble closely the line found in the late spread of the local lesions into the uninoculated tissues of the inoculated leaf. This may be seen by comparing Figures 2 H and 2 I. The leaf shown in Figure 2 I was collected 14 days after inoculation of one-half of the leaf by rubbing. The original lesions had fused and become indistinct and the pattern had spread into the opposite side of the leaf, its periphery being sharply marked and similar in appearance to the contour in Figure 2 H.

The similarity between the local lesions on *N. tabacum* described in this paper and the necrotic local lesions previously observed on other hosts inoculated with the same virus is shown in Figure 3. The lesions shown in this figure were produced by rubbing half the area of each leaf with extract of mosaic plants. The first three photographs in this figure (A to C) show leaves of Turkish tobacco with local lesions stained in iodine. The leaf shown in Figure 3 A represents an infection two days after inoculation; that in Figure 3 B three days after inoculation; and that in Figure 3 C four days after inoculation. The remainder of the photographs show necrotic local lesions resulting from the establishment of the same virus in the leaf tissues of other hosts: (D) *Nicotiana glutinosa*, (E) *N. rustica*, (F) *N. langsdorffii*, (G) *N. sanderae*, (H) *N. acuminata*, (I) *N. glutinosa*  $\times$  *glauca*, (J) *Capsicum annuum* variety Anaheim Chili, (K) *Solanum pseudocapsicum*, (L) *S. melongena* variety Black Beauty, (M) *Nicotiana alata*, (N) *Datura stramonium*, and (O) *Phaseolus vulgaris* variety Early Golden Cluster.

The lesions in Figure 3 A are the smallest shown in the figures of this paper, although smaller lesions were observed in leaves collected as early as a day and a half after inoculation. Small lesions at pin punctures were

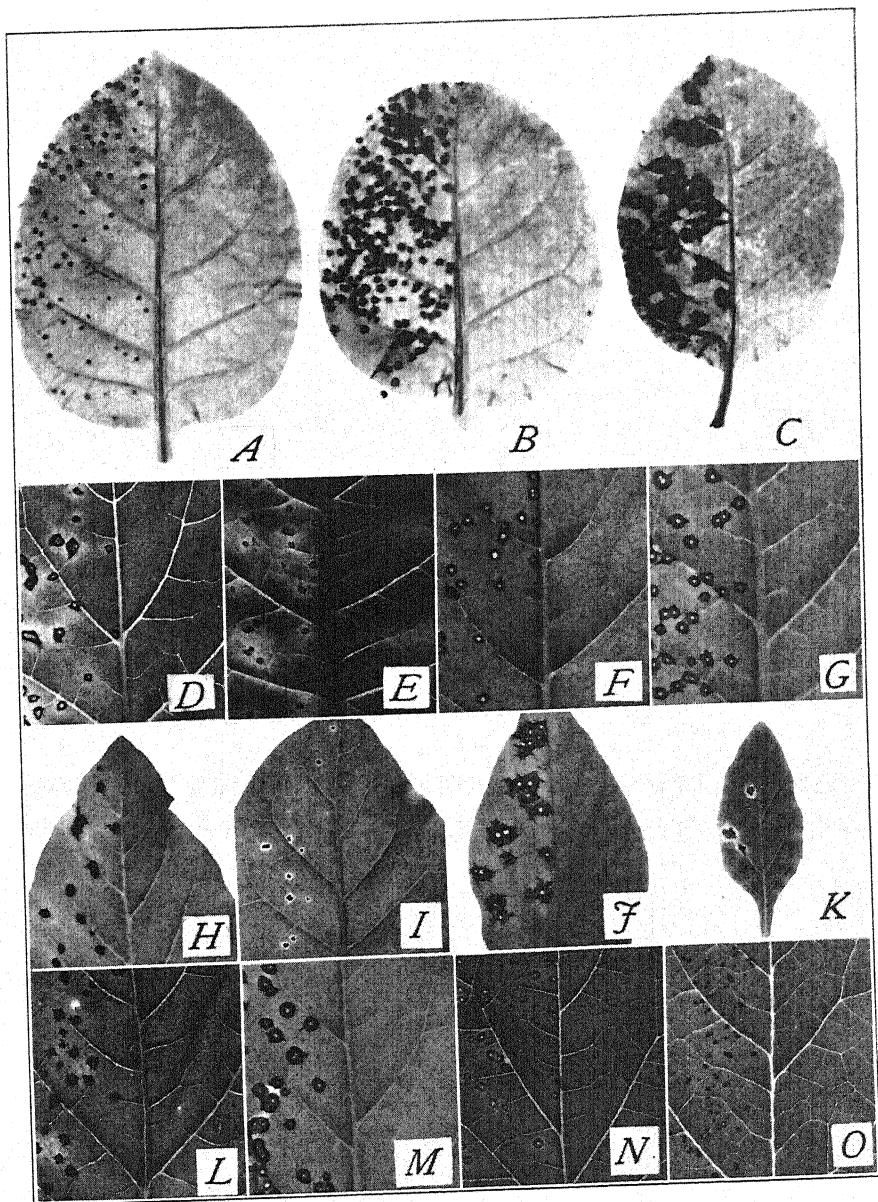


FIGURE 3. Leaves of *N. tabacum* variety Turkish stained in iodine to show local lesions (A) two days, (B) three days, and (C) four days after inoculation. Portions of living leaves showing necrotic local lesions in (D) *N. glutinosa*, (E) *N. rustica*, (F) *N. langsdorffii*, (G) *N. sanderae*, (H) *N. acuminata*, (I) *N. glutinosa*  $\times$  *glauc*, (J) *Capsicum annum* variety Anaheim Chili, (K) *Solanum pseudocapsicum*, (L) *S. melongena* variety Black Beauty, (M) *Nicotiana alata*, (N) *Datura stramonium*, and (O) *Phaseolus vulgaris* variety Early Golden Cluster.

especially interesting, because they did not surround the wounds, but involved tissues on one side, as would be expected if only one or a few cells were originally infected in each case. An example of this is well shown in Figure 2 A.

#### PRESENCE OF VIRUS IN LESION AREAS

It appeared probable that the lesions herein described corresponded with areas in which virus had been increasing in concentration. An experiment was performed to determine whether areas which contained measurable quantities of virus could be chosen by appearance alone. Four pin punctures were used to inoculate virus into each leaf of a number of Turkish tobacco plants. When yellowish lesions became visible around some of these punctures, a pair of tissue samples was taken from each leaf. One sample in each pair contained a puncture surrounded by a yellowish lesion, and the other a puncture surrounded by normal green tissue. The samples were squares of leaf tissue about three-quarters of an inch on a side. All samples were tested on the sixth, seventh, or eighth day after inoculation. The leaves at this time corresponded in appearance with that in Figure 1 A. Each sample of tissue was wrapped in a small square of cheesecloth, and pounded to express its juice. The cloth moistened with the expressed juice was then rubbed over a single leaf of *N. glutinosa*. On this plant the number of lesions produced upon inoculation by rubbing has been shown to be dependent upon the concentration of virus in the inoculum (2, p. 47-53). Virus measurements were made of 25 samples showing lesions, and of the same number of samples showing no lesions.

The results of the 50 measurements are given in Table I. No necrotic lesions developed on the leaves inoculated with juice from the 25 samples showing no yellowish lesions; but when inoculations were made with juice

TABLE I  
NUMBER OF LESIONS PRODUCED ON NICOTIANA GLUTINOSA LEAVES BY THE  
INOCULATION OF EXTRACTS FROM YELLOWED AND NORMAL GREEN AREAS  
SURROUNDING INOCULATION PUNCTURES IN LEAVES OF TURKISH  
TOBACCO

Yellow areas	Corresponding normal green areas	Yellow areas	Corresponding normal green areas	Yellow areas	Corresponding normal green areas
315	o	185	o	280	o
308	o	204	o	360	o
325	o	192	o	318	o
160	o	188	o	176	o
64	o	134	o	324	o
138	o	168	o	384	o
86	o	245	o	496	o
120	o	121	o	585	o
		225	o		

Total number of lesions on leaves in yellow area series 6101; average 244.

from the 25 samples which showed yellowish areas around the puncture, more than six thousand necrotic lesions were found on the inoculated leaves, and none of such leaves failed to show a considerable number of necrotic lesions. These results appeared to indicate a correlation between development of a yellowish halo and high virus content of the tissues around each point of infection under the conditions of these experiments.

#### DISCUSSION

The examination of local lesions produced by the virus of tobacco mosaic in leaves of *N. tabacum* constitutes a useful method of investigation. The behavior of the virus immediately after introduction into the tissues of the host plant, previously studied by quantitative methods (3), is more graphically shown by iodine-stained specimens, which also give information about the number and position of points of entrance of the virus.

It should not be assumed that the pattern in stained leaves coincides exactly with the distribution of virus at the time of collection of a specimen. It is probable that the pattern more nearly represents the location of virus somewhat earlier. Nevertheless, knowledge of the nature of the pattern suggests tests which may show more precisely the tissues involved in the movement of the virus through the plant.

#### SUMMARY

It has been known for some time that tobacco mosaic produces necrotic local lesions in some of its hosts. No necrotic local lesions appear on inoculated leaves of *N. tabacum*, however, and it has usually been considered that no local lesions of any kind develop at the site of inoculation in this plant.

The present paper describes yellowish local lesions which were frequently found to develop at the points of infection in green leaves of *N. tabacum* variety Turkish inoculated with tobacco mosaic extracts. Staining inoculated leaves with iodine rendered the lesions conspicuous and revealed the points of infection even when environmental conditions had been such that the yellowish lesions were indistinct or not visible in the living leaf. It was found that under the conditions of these experiments the formation of these lesions indicated that virus was present locally in high concentration. The method described for the study of these local lesions in *N. tabacum* allowed the recognition of invaded tissues in old inoculated leaves not affected by the well-known systemic symptoms of tobacco mosaic.

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## A MORPHOLOGICAL STUDY OF GLADIOLUS

NORMA E. PFEIFFER

Because of its attractiveness as a garden plant, the genus *Gladiolus* L. has received much attention in literature, chiefly from the systematic and horticultural points of view. An occasional study has thrown light on a morphological phase and more recently some physiological experiments have been carried out with *Gladiolus* as a test plant. It seemed worth while to organize the scattered pieces of work as closely as possible and to carry on a study of the internal structure, especially in the course of development of the inflorescence.

### HISTORICAL

The history of the genus from the systematic point of view has been considered in a general account by Beal (2). He has also included notes on the introduction and early cultivation of the wild species, originally known as "corn flags," and the development of cultivated forms from native plants of the Mediterranean region and of Africa, especially the region of the Cape of Good Hope. It may suffice here to call attention to the position given to the genus by Pax in Engler and Prantl (17) under Gladioleae in the family Iridaceae. The genus is representative of the family in these features: plants herbaceous; leaves equitant; flower axis terminal; flowers tubular with six-parted perianth differentiated into two sets; stamens three with extrorse anthers; inferior ovary producing a loculicidal capsule with many anatropous seeds. It is distinctive among the genera in that it shows the following combination of characters. The spathe is always one-flowered in the spike; the style branches are simple rather than divided; the more or less zygomorphic flower has a curved funnel-like perianth, with the segments as long as, or longer than the perianth tube. Zygomorphy is seen in the production of two lips, into the arch of the upper of which the stamens on long curved filaments and the slender style with three-lobed stigma become fitted by the time of anthesis.

There is much literature as to the differentiation of the perianth segments into the two lips (5, 8, 25, 26). At anthesis these segments may exhibit differences in size, shape, coloring, and markings. There are two possibilities as to the final differentiation of the three members in each perianth set. The arch of the upper lip may be formed by a member of the outer perianth set and the lower lip be due to the cooperation of the other two outer perianth parts and one inner (5, 8, 9, 26). Or the uppermost segment may belong to the inner perianth, when it follows that the lower lip consists of an outer segment flanked by two inner perianth parts (8, 9, 25, 26, 29). In wild species, the latter seems to be the more common according to

most accounts, but one arrangement or the other is characteristic and constant within the species; in garden hybrids both arrangements may be found (9, 25).

A striking feature in most species lies in the fact that although the flower buds are in two ranks in the spike, the blooms shift position so that at anthesis the effect of a more crowded single row is given. The torsions by which this result is attained have been the subject for several investigations of which the most recent is that by Haeckel (8), who discusses the earlier reports. Her study includes not only a consideration of the inflorescence but also dorsiventrality in the flower. By using darkness and one-sided illumination she finds that light is not a factor. By the use of the clinostat she obtained flowers with a radial outer perianth and with an inner perianth (of which two parts normally contribute to the lower lip in the species used) showing all gradations between a lip and complete lack of such differentiation. The stamens were arranged radially about a central style. Gravity is then the factor which determines the position and differentiation of the perianth member and the movement of the style and of the stamens from their original position opposite the outer perianth parts. This might be indicated also by the field observations of Marloth (16) who found the flower spikes of *G. cardinalis* Curt. growing in a hanging position on moist precipices and grassy ledges in Cape Colony. On these inverted spikes, the flower parts assumed the same relation to gravity as in upright spikes. In young spikes cut and placed in an upright position, he found the lower lips of the unfolding flowers directed away from the spike tip rather than toward it as in the pendent spikes.

The bending and twisting in which the inflorescence axis, the axes of individual flowers, spathe parts, perianth parts, stamens, and style are all concerned in greater or less degree, are illustrated diagrammatically by Urban (26), Troll (25), and Haeckel (8). Troll is inclined to believe that the variation in garden hybrids as to the contribution to the lower lip by one or two members of the inner perianth set is a result of degree of torsion, rather than differences in original insertion of parts.

Other investigations will be considered in connection with the results of morphological study of the internal structure.

#### METHODS

This work was started in the fall when newly-harvested corms of *Gladiolus* are apt to be dormant. Hence treated corms<sup>1</sup> of two varieties, Halley and Alice Tiplady, with a little additional material of varieties Maiden's Blush, Remembrance, and Souvenir, were used. The major lots of *Gladiolus* had been obtained from Mr. A. Ludeke of Castle Hayne, North Carolina,

<sup>1</sup> The corms were obtained through the courtesy of Dr. F. E. Denny after treatment by methods previously reported (4).

and consisted of Halley variety harvested on August 6 and of Alice Tiplady variety harvested on August 19 and 20. After treatment on September 28, the corms were planted in flats and held in the basement at a relatively low temperature till shoots began to appear, when they were removed to the greenhouse. Superficial examination to note evidences of activity prior to actual elongation of the shoot, and continued observation thereafter led to killing and sectioning of buds at intervals of a few days to a week from October 17 through December for Halley and into February for Alice Tiplady. The buds were usually trimmed at the sides for longitudinal sections. The most effective orientation was in the plane parallel with that in which the buds of the corm lie. These shoot buds were killed in formalin-alcohol-acetic acid mixture, sectioned and stained in safranin and crystal violet, safranin and light green, or Haidenhain's haematoxylin. For later developmental stages, particularly in the individual flower, chromo-acetic acid mixtures, with or without osmic acid, including Flemming's weaker fluid, were used. Generally material was killed about 1:00 P.M., the time of day ordinarily considered most favorable for mitotic division in plants, although Winter (28) reports in a detailed study of the roots that there appears to be no definite mitotic periodicity in this organ in *Gladiolus*.

## RESULTS

### EXTERNAL APPEARANCE

In this genus, the corm consists of the lower swollen internodes of the axis which produce a storage organ capable of reproducing the plant. This basal portion is covered during its formation by the lower part of the leaves which become dry and scarious by the end of the season and persist as covering for the storage organ (Fig. 5). Owing to the strongly two-ranked arrangement of the equitant leaves in *Gladiolus*, the buds lie on alternate sides in one vertical plane which bisects the corm. Of these buds the largest is the uppermost, nearest the base of the old elongated inflorescence axis. It usually produces a taller shoot than do succeeding buds, but if it is injured or removed early, the second bud takes the lead and produces practically as strong a shoot. Even the smallest of these axillary buds may develop although they are ordinarily inhibited by the growth of those above them. Furthermore, unexpectedly large shoots may be produced in darkness where it is obvious that the new structure must depend entirely on food stored in the corm. One specimen in peat moss in darkness produced a shoot 54 cm. tall, in which the dissected bud showed a blasted flower spike with possibly seven flowers started.

The arrangement of parts in the shoot bud is in line with that in the corm producing it; the very young leaves bear the same relation to each other and the small buds in their axils lie in the same plane as the larger buds related to the leaves of the main corm. In resting condition the large buds are slightly depressed on the surface of the corm.

Most of the shining scarios scales had been removed from the corms so that only the innermost, directly over the buds, were left. During the frequent examination for the first external signs of activity, it was seen that with the swelling of the tissues of the bud, these scales were cracked. The young leaf rudiments, present in the main bud, pushed through at

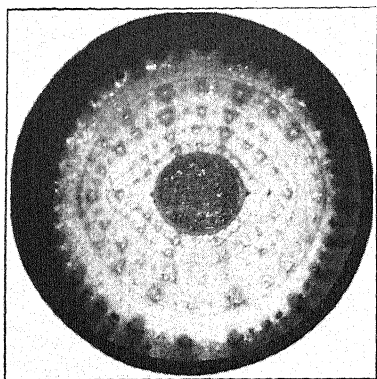


FIGURE 1. Shoot cut across near base of floral axis (center); midribs of the leaves have alternate positions, with that of the innermost leaf at the left, of the next at the right, etc. Extensions of the veins of the outermost leaf appear as dark lines in the marginal zone which is due to swelling of the new corm. Magnification 6.

first as a sort of cone which was broken as successive leaves outgrew those that preceded them. The leaves are of two sorts, the lower or sheath leaves and the upper or true foliage leaves, as described early by Irmisch (11) and recently by Geiger (6). The basal leaves are very much shorter and smoother; of the five produced (Fig. 2), usually only two are permanent and hence recognizable at maturity of the plant. The three smaller lower ones dry and are lost in development. In Halley there is a fair production of anthocyanin in the sheath leaves, more markedly in the lower ones, whereas Alice Tiplady leaves are green throughout. In contrast with these short smooth leaves, the true foliage leaves are extended into a conspicuous portion which by lateral flattening produces a limb. In this limb the veins project on both surfaces giving it a characteristic

ribbed effect. Basally the leaves appear like a series of approximately concentric layers, lacking uniformity in thickness in the individual. This may be seen in a section near the base of the shoot (Fig. 1) where the wider midvein of one leaf appears at the left, while that of the succeeding leaf is at the right. Higher up the margins of the foliage leaf are separated along a line opposite the midrib (Pl. III, 36). The limb is produced by fusion of the tissue beginning with the midrib (Pl. III, 35). At the level where all the leaf is limb with no part ensheathing the shoot, there is an interesting double structure with the effect of a midrib entirely different in position from that recognized below (Pl. III, 34). This has been designated the pseudo-midrib, and is a feature even in *Gladiolus* species which produce tetragonal or terete leaves differing from the sword-like form of the varieties here considered (1). There is much sclerenchyma tissue associated with the numerous fibro-vascular bundles which gives a firm character to the long narrow limbs.

More frequently than not in this material elongation of the shoot pre-

ceded formation of roots. Halley variety produced roots more quickly than Alice Tiplady. In the former variety on October 25 when flats were removed to the greenhouses, 17 out of 38 shoot-bearing corms showed some root development, while in Alice Tiplady only 2 out of 13 corms with shoots showed roots.

#### INTERNAL STRUCTURE

Of greater importance is a study of the internal structure in connection with the external appearance. The significance of the corm has been considered in earlier accounts as brought together by Geiger (6) and this structure was not further studied here.

*The shoot.* Before the main bud breaks through the tough protective scales, the young meristematic tip is covered by seven or eight leaf rudiments. With the uptake of water and the beginning of activity these young leaves elongate, pushing through the confining scales. In different batches of material, numerous mitoses were found in the bud tissues 12, 16, and 17 days after the corms were started (Pl. I, 8). This was coincident with external evidence of activity. Undoubtedly mitotic divisions would have been discernible with earlier microscopic examination. As the leaves elongate, one may observe zones of cells actively dividing and others above these in the phase of elongation. Additional leaf primordia are cut off from the meristematic tip. As seen in sections cut parallel with the diameter of the corm in which the buds lie, the leaves already give a two-ranked effect in the orientation of their pointed tips which are directed to the right or left of the median plane in successive leaves (Pl.

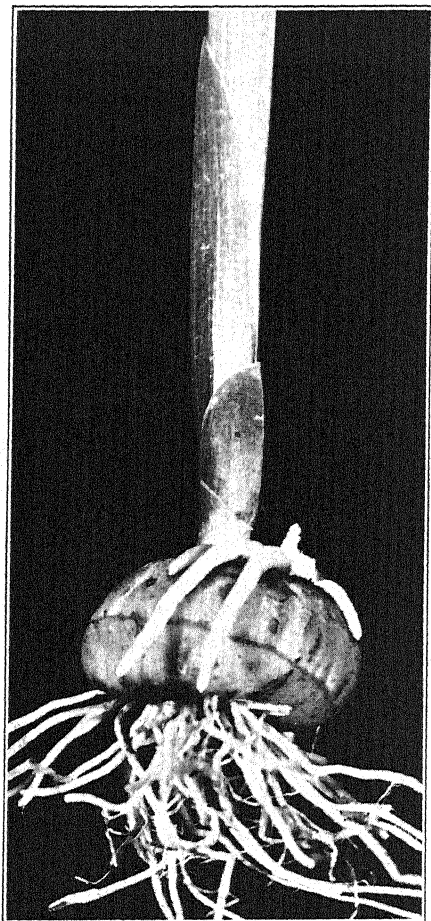


FIGURE 2. Young plant (Halley) bearing two similar shoots. The outer leaves have been dissected from the one on the right to show the upright floral axis and new corm, with four young smooth contractile roots (contrasting sharply with older fibrous roots of the mother corm). The shoot at the left shows four sheath leaves and the oldest of the foliage leaves. About 60 days after planting.

I, 8; II, 14). The lower internodes in the bud develop more rapidly than the upper ones with marked expansion in all directions while there is still little extension of the upper internodes.

*Flower spike.* In the mature plants there are characteristically five or six foliage leaves. After the primordia of these have been cut off, there is vertical extension of the central region (Pl. I, 9; II, 15). This is accompanied

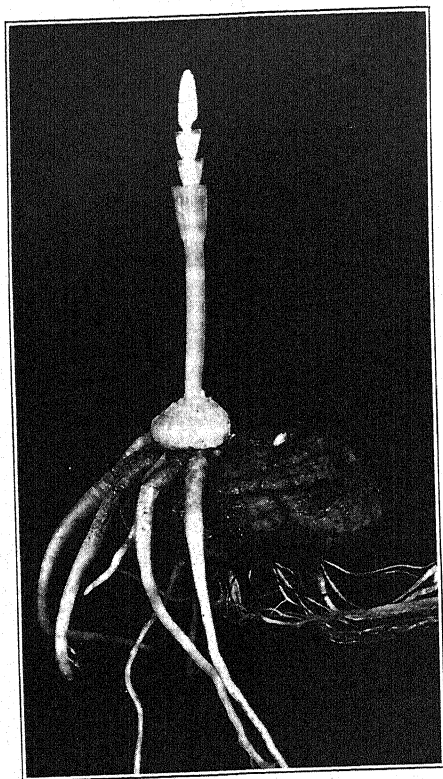


FIGURE 3. Young plant (Alice Tiplady); the leaves have been removed from the young corm; those of the floral axis, except the uppermost which enclose the flower spike, have been trimmed. The contractile roots show the older wrinkled thinner region and the intermediate smooth swollen region.

by gradual differentiation of the floral spike, which is terminal. Early stages in the elongation of this axis are more readily detected in microscopic study. By the time such elongation is readily distinguished by the naked eye and becomes measurable by gross method (Fig. 2) differentiation of flower primordia has already occurred. Acropetal development results in a spike of a variable number of flowers arranged alternately in two rows (Pl. I, 10, 11, 12). This spike is short and remains compact with the primordia close together until most of the rudiments are formed (Pl. I, 10; II, 19). Differentiation has begun in the tissues of the organs in the lower-most flower by the time the flower spike elongates markedly (Pl. I, 11, 12). At the same time the leaf-bearing portion of the axis below the flower spike is increasing in length so that the internodes are distinguishable without magnification by the time the bract-enclosed spike is three or four millimeters long. Some variation in degree of stoutness as well as length of spike is found. The two varieties chiefly used are of the stouter type (Fig. 3), while Maiden's Blush, used for supplement-

ary study, is more slender. Alice Tiplady produced a spike of fewer flowers, six to nine, while Halley produced as many as thirteen to sixteen. Later growth of the axis is shown in Figure 4.

*Differentiation of the flower.* In any of these varieties the differentiation

of the floral members may be followed in longitudinal sections of the whole spike (Pl. I and II) and in cross sections of individual flowers (Pl. III, 24, 25). Low papillae are produced which soon differentiate into the bract eventually forming the outer spathe member and the rounded flower primordium (Pl. I, 9; II, 15 to 18). The first prominences to develop on this are the rudiments of the three stamens (Pl. I, 11; II, 19, 20). The inner spathe member, reported by Payer (18) as a fusion of two eminences, becomes evident at about the same time. Very soon a region of meristematic tissue below the stamen set develops as a preliminary stage of the floral envelopes (Pl. I, 11, 12; II, 21), which differentiate with more rapid early growth of the calyx parts (Pl. III, 24). The central region between the stamen primordia becomes distinctly broader with the formation of three carpels (Pl. I, 12, 13; II, 20, 22; III, 25). This sequence of development differs from Payer's early account (18) for *G. communis* L., which gives the order as sepal, petal, stamen, and carpel sets.

The history of stamen development is the usual one, with formation of wall layers, tapetum, and sporogenous tissue (Pl. III, 23, 24). The pollen mother cell has been used by Lawson (14) in his study of the origin of the multipolar spindle.

Ovules are produced in two rows in each of the three chambers of the pistil, with great symmetry (Pl. I, 13; III, 25). These lie at first with their long axis in a horizontal plane, but gradually shift with development. At

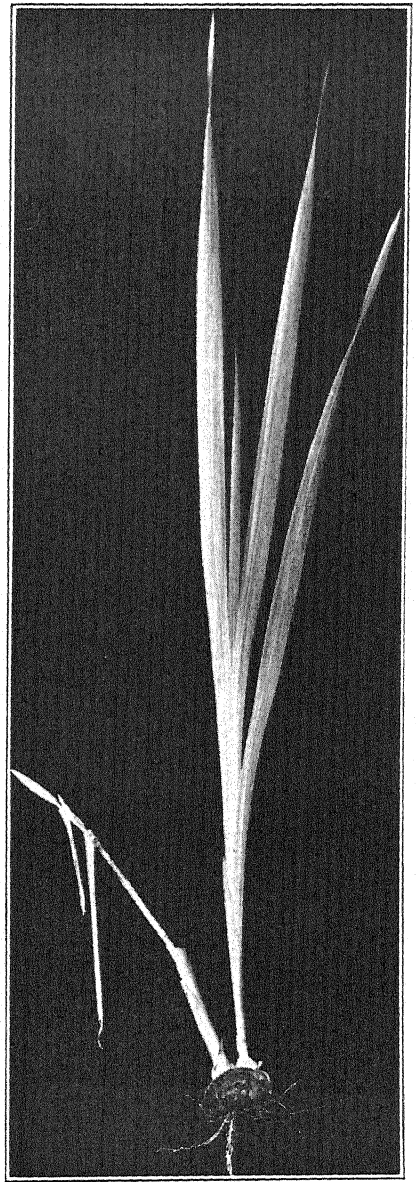


FIGURE 4. Older plant; the leaves of the shoot on the left have been trimmed or turned back to show the flower spike.



## EXPLANATION: PLATE I

Photomicrographs of longitudinal sections of *Gladiolus* floral axes. Magnification is 15, in 8 to 12, approximately 27 in 13.

8. Upper portion of corm (Alice Tiplady) with active main bud, still vegetative in character, with mitoses in young leaves and stem; about 19 days after planting. Note evidence of arrangement of leaves in two ranks; outer scales have been removed.

9. Very young floral axis (Halley) about 34 days after mitoses were observed. Two flower primordia show the production of the outer spathe.

10. Upper part of young floral axis (Halley) showing elongation of flower spike and nodes below it; about 50 days after planting.

11. Flower spike (Halley) showing acropetal development and differentiation of individual flower. The small secondary flower spike on the lower right duplicates the development of the main spike, with fewer flowers. A similar spike is cut tangentially at the left.

12. Older flower spike (Halley) with 17 flower primordia. In the lowermost, the stamens are well differentiated and the carpel chamber closed. Secondary flower spikes occur in the axils of the leaves just visible at the margins; 79 days after planting.

13. Individual flower (Maiden's Blush); outer spathe, incomplete at right; inner spathe chiefly at left, extended about base of flower at right; inferior ovary with ovule primordia visible in one chamber; short perianth parts; and relatively large stamens with sporogenous tissue.

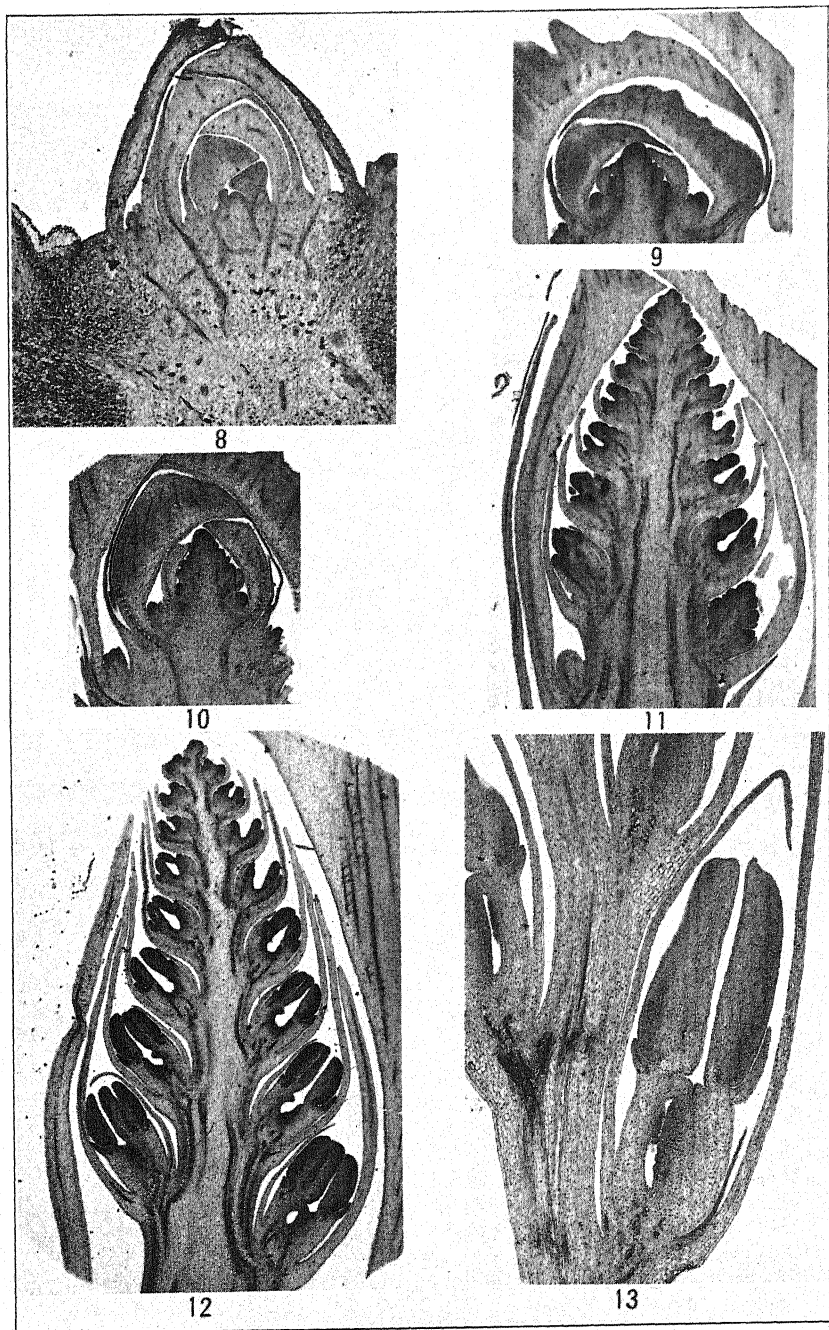


PLATE I—MORPHOLOGY OF GLADIOLUS

## EXPLANATION: PLATE II

The drawings were made with the aid of a camera lucida. Magnification 32 in 14 to 19; 50 in 20 to 22. *r*, papilla representing first stage in production of flower; *o*, outer spathe; *i*, inner spathe; *s*, stamen; *p*, perianth; *c*, carpel.

14. Vegetative stage of stem apex, with leaf primordia.

15. Very young stage of the flower spike, with first papillae which will develop into flowers.

16, 17, 18, 19. Successive stages in the development of the flower spike, showing acropetal production of flower primordia and their differentiation. The upper leaves of the floral axis, with buds in their axils, enclose each spike.

20, 21, 22. Differentiation of the individual flower, within the spathe members, showing successively the production of stamen rudiments, perianth, and the broadening and deepening of the central region in carpel development.

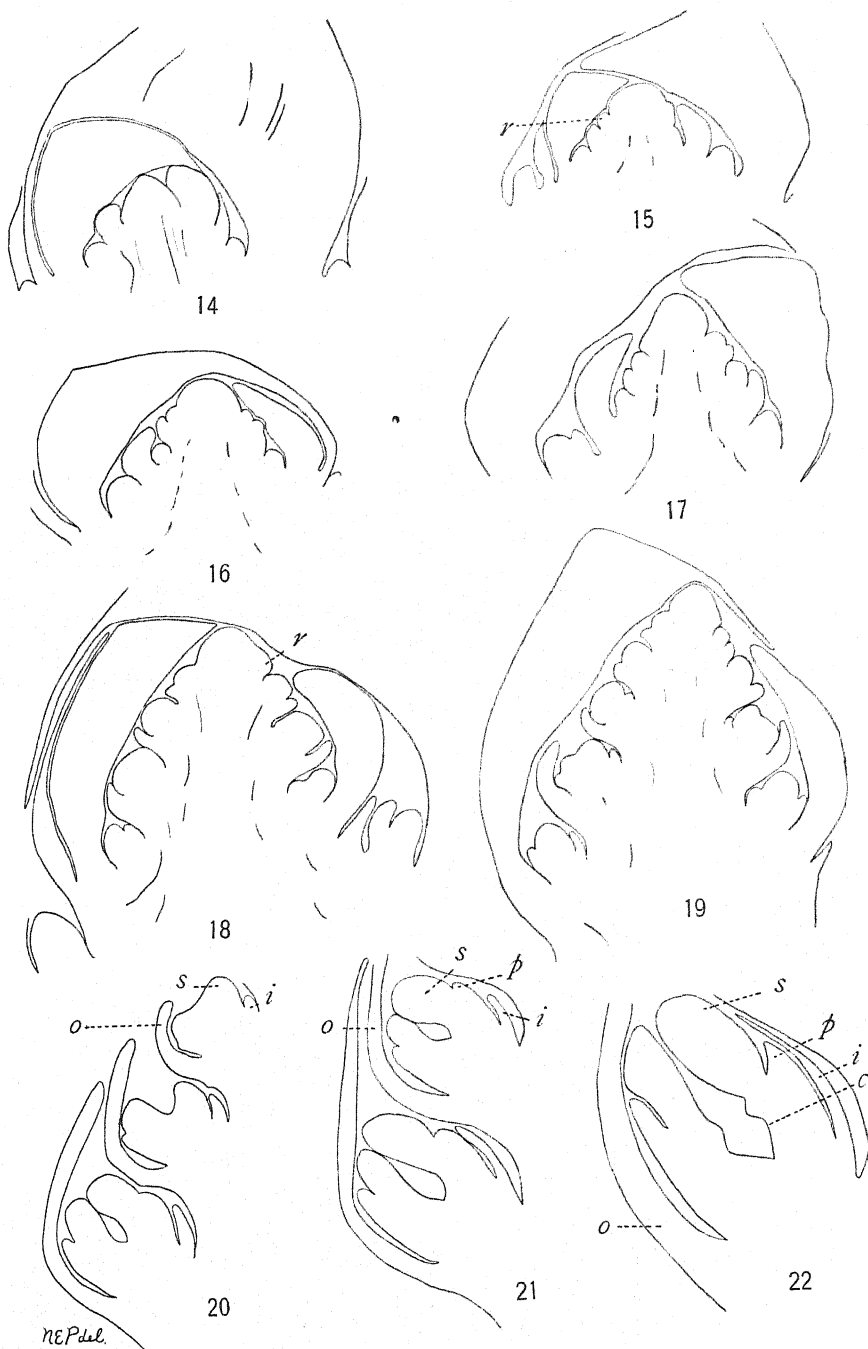


PLATE II—MORPHOLOGY OF GLADIOLUS

## EXPLANATION: PLATE III

23. Portion of a cross section of an anther, showing a single microsporangium with sporogenous cells, tapetum and wall layers. Magnification, 202.

24. Cross section of a young flower at upper level: spathe removed; perianth consisting of three sepals with stamens opposite them, and three less developed petals; tips of style branches in the center. Magnification, 34.

25. Cross section of the same flower near the base. Part of outer and all of inner spathe members surround the young ovary with its ovules. Magnification, 34.

26. Longitudinal section of a young ovule with megaspore mother cell, nucellus, outer and inner integuments in early stage of development. Magnification, 345.

27. Longitudinal section of micropylar region of young embryo sac, showing synergids and egg; nucellar layer is intact. Magnification, 345.

28. Longitudinal section at a later stage, when the synergids have penetrated the nucellus. Synergid beak (filiform apparatus) projects slightly into the micropyle. Magnification, 345.

29. Longitudinal section at a still later stage, when the beak projects far into the long narrow micropyle; walls of the tip region of the synergids are differentiated; membranes between egg and synergids are not distinguishable. Magnification, 345.

30. Longitudinal section of embryo 5 or 6 days after pollination, showing basal cell, suspensor region and portion that will produce embryo. Magnification, 202.

31. Embryo 10 days after pollination. Portion of old synergid at upper left of basal cell. Magnification, 202.

32. Embryo 12 days after pollination. The beginning of development of the lateral stem-tip results in asymmetry. Magnification, 202.

33. Embryo 21 days after pollination, about a week before seed is mature; cotyledon, stem-tip with first leaf, and root-tip are well developed, while basal cell remains conspicuous. Magnification, 31.

34, 35, 36. Cross sections of a leaf at three levels, upper blade with double structure, pseudo-midrib *s* and midrib *m*; region with part blade-like, part clasping the stem; and base with all of leaf sheathing the stem. Diagram of vascular bundles with sclerenchyma.

37. Portion of vascular cylinder of contractile root; *e*, endodermis; *p*, pericycle, *x*, xylem; *x'*, metaxylem; *f*, phloem. Magnification, 360.

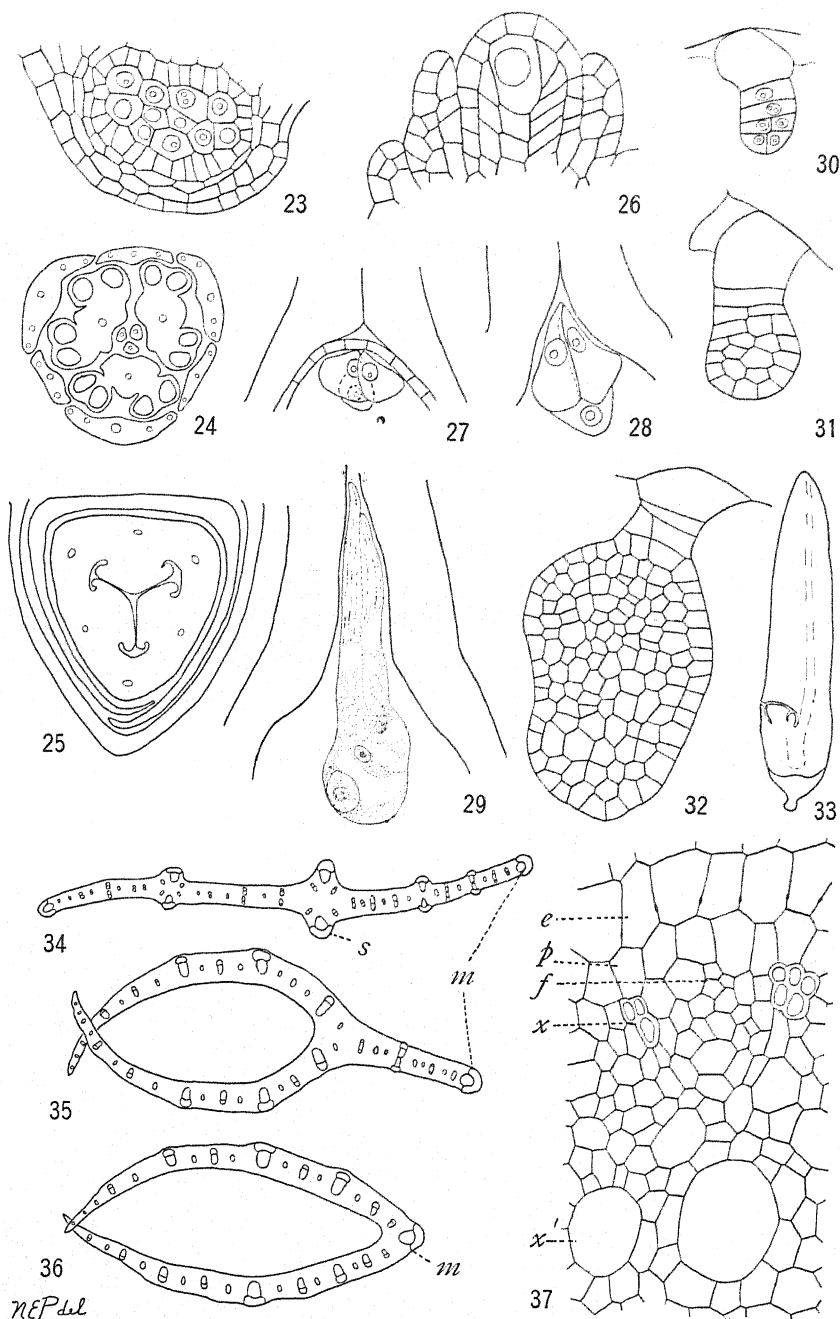


PLATE III—MORPHOLOGY OF GLADIOLUS

the stage in seed production when the wings become clear, they are in a more or less pendent position, with the two sets in a single chamber interleaved in one series. When the fruit splits from the top downward, the two rows are again carried apart by dehiscence of the capsule along the suture between them.

The ovule develops characteristically for monocotyledonous forms and produces two integuments with a very long micropyle at maturity. The outer integument consists of at least five layers of cells, the inner of but two. This is already indicated in as young a stage as is shown in Plate III, 26, where the megaspore mother cell has developed. (At this time there are microspore tetrads in the anthers.) The functioning of the mother cell was not observed. A characteristic eight-nucleate embryo sac is produced. Here the antipodal nuclei at the chalazal end are conspicuously large and the equally striking synergids, after resorption of the nucellus layer, project far into the micropyle in beaked form (Pl. III, 27, 28, 29). The resulting filiform apparatus previously reported for *G. segetum* Ker. (23, 7) is supposed to be cellulose and to play a chemotactic rôle in secreting a glucose-containing substance (7). Such structures are found in other angiosperms and their association with long narrow micropyles led to the assumption that they are "of assistance in the progress of the pollen-tube" (3, p. 94). In *Gladiolus*, the projections are relatively resistant, and although there is evidence of their breaking down after fertilization, they sometimes persist until the embryo consists of seven or more cells.

*The seed.* After fertilization, transverse divisions of the fertilized egg result in a single row of five cells. Then vertical walls appear in the division of the terminal cells (Pl. III, 30) and in seven to ten days a spherical embryo results (Pl. III, 31). The basal cell is large and the suspensor region is very short. Meanwhile division of the endosperm nucleus which was near the chalazal end of the embryo sac has produced many free nuclei arranged parietally. Later fine endosperm walls come in, with centripetal growth. In such a stage as that shown in Plate III, 31, there may be three layers of endosperm cells within the sac. The embryo loses its spherical shape by elongation, and a lateral stem-tip develops (Pl. III, 32). The regions of cotyledon, stem-tip, and root are rapidly differentiated so that they are distinct and well organized in three weeks (Pl. III, 33). These stages were obtained in self-pollinated Maiden's Blush. Attempts to self-pollinate Remembrance flowers gave a low proportion of seeds with embryos, many with endosperm but no embryo, and an even larger number of ovules which failed to develop at all. As is known, some varieties are not self-fertile (15).

At maturity, the winged seed contains a relatively large embryo surrounded by endosperm cells with thick hemicellulose walls. The nucellus has disappeared except for a conspicuous pavement of differentiated tissue at the chalazal end, while the originally two-layered inner integument has

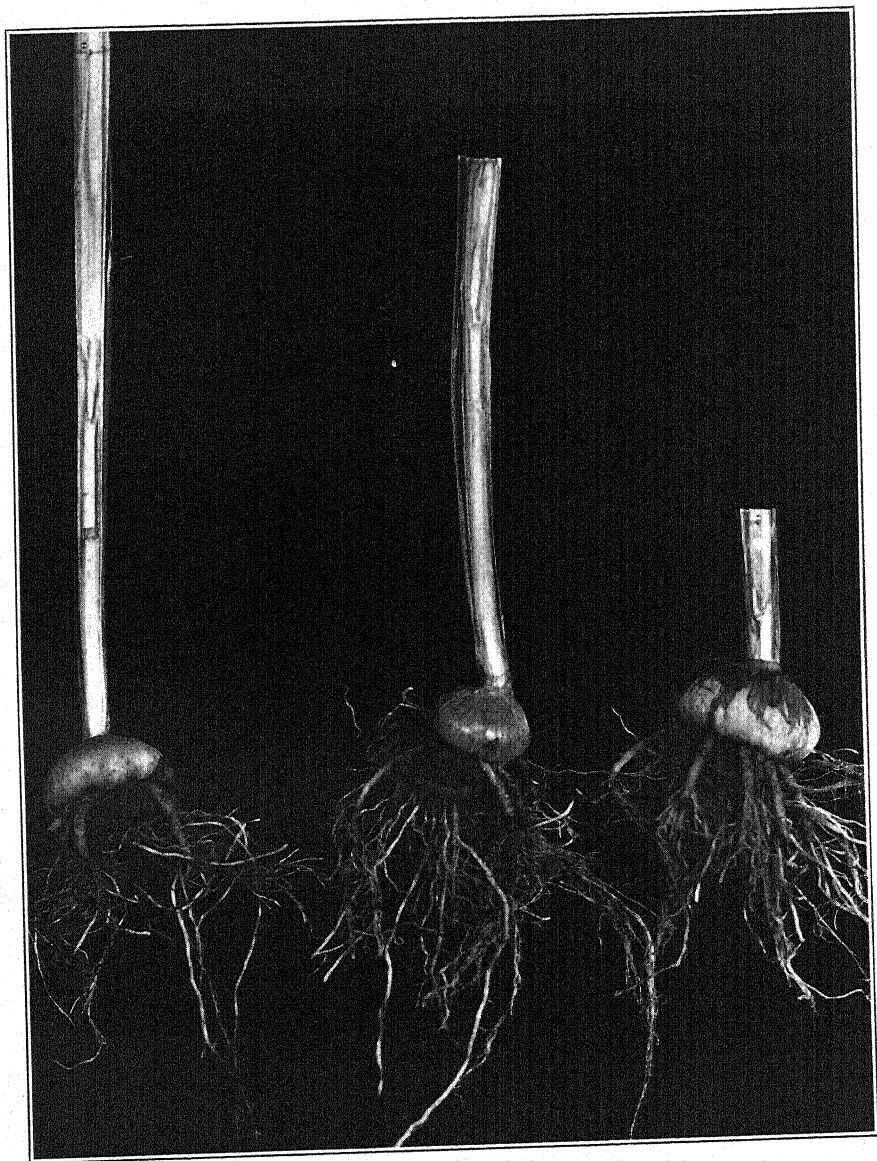


FIGURE 5. Older plants; the front half of the leaves of each shoot has been removed to show the blasted flower spikes; floral axes show different heights. The new corms are well developed, with leaves becoming scarious.



suffered gradual compression. The innermost cell layer of the outer integument increases tremendously in the radial dimension during embryo development and at maturity is very conspicuous because of its size and the thickening of the cell walls.

*New corm.* During the development leading to the flowering stage as recorded above for the central bud, there has been further increase in size of the lower internodes of the stem which give rise to the new corm for the current season. This is the swollen portion about which the bases of the leaves are closely appressed. Even before the inflorescence axis appears to the naked eye to be elongated, this new storage organ may become over a centimeter in diameter, with rapid accumulation of much food reserve.

*Contractile root.* At about this time, the first of the fleshy roots which are known as contractile roots appear at the base of the new corm (Fig. 2). Whereas the usual basal roots numbering from about 20 to 70, according to the size and vigor of the corm, have served to anchor and provide for the necessary uptake of water for the original organ, these new thick white roots, as much as 0.7 cm. in diameter (Fig. 3), seem to have additional functions. They have numerous fine hairs on the surface suggesting absorption. They undoubtedly serve to anchor the body and in common with roots of the same sort as reported for *Lilium Martagon* (20) and other forms (1, 21, 22), to bring it to an appropriate level in the soil. Some are produced where they cannot function in this way, but these often project themselves for short distances into the old corm. These roots in the young stage of any portion seem plump and smooth on the surface, as if the cells were much stretched. As the region matures there is longitudinal shrinking which throws the surface tissue into obvious wrinkles, while it serves to bring the corm to a greater depth in the soil (Fig. 3 and 5). There is also a decrease in diameter with maturing of the root. In addition to the rôles of absorption, anchorage, and determination of location in the soil, there is no doubt that the swollen roots, developing to the number of ten or so, serve as temporary storage organs since they contain much sap and food material. Several roots weighed and dried at room temperature until of constant weight showed the water content to be 93 per cent of the fresh weight. Microchemical tests on roots of various ages showed the presence of a little fructose, more glucose, much dextrin and other reducing substances, with variable amounts of protein substances; no starch was found.

Longitudinal sections show the presence of a shallow root cap and meristematic tip. Cross sections near the tip (Fig. 6 A) indicate early differentiation of tissues with epidermis (from which short fine root hairs arise), wide cortical region made up of outer larger and inner smaller cells, endodermis, and pericycle layer limiting the central cylinder. In the latter are protoxylem points about 10 to 25 in number (Fig. 6 A and B), at first with a single vessel, but frequently with as many as eight well-lignified cells per

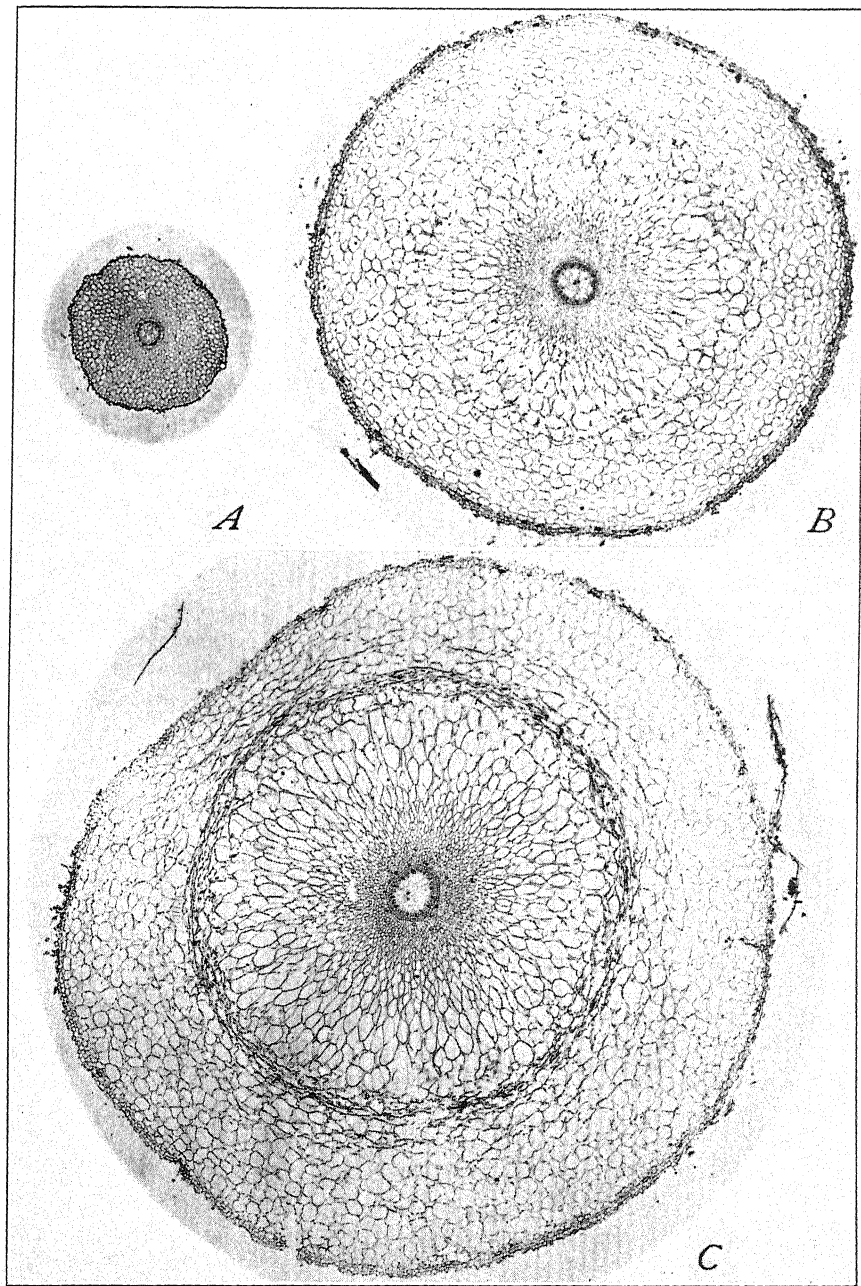


FIGURE 6. Cross sections of contractile root; magnification 15. A. Growing region near the tip; the chief tissues are differentiated. B. Intermediate region; root hairs have collapsed, epidermis and hypodermis are sharply differentiated; strains are evident in cortical cells, although the exterior of the root appears smooth. C. Older region where the wrinkled exterior indicates longitudinal contraction and the decreased diameter, radial contraction; this contraction results in several layers of crushed cortical cells.

group in old roots. Usually on the same radius with the protoxylem, occasionally on alternate radii, too, there is a single metaxylem vessel of large caliber. Small phloem patches alternate with the protoxylem groups. Centrally there is a persistent pith, eventually lignified. A portion of the vascular cylinder is shown in Plate III, 37.

Older roots show that definite changes occur in the different tissues both in the chemical constitution of walls and in form and size of cells. The



FIGURE 7. Strip of hypodermis dissected from a contractile root at about the level shown in Figure 6 C; the radial (lateral) wall is seen in outline as a double wavy line *a*; the folds in the wall give a misleading spiral effect *b* when the face of this lateral wall is seen obliquely. Magnification 315.

outer wall of the epidermis and all cortical walls become suberized while the radial walls and the outer wall especially of the well-differentiated hypodermis give a slight reaction with phloroglucin, indicating slight lignification. As to form, the originally much elongated cells undergo contraction so that the walls assume a wavy character which is most marked in the heavy walls of the hypodermis (Fig. 7) and endodermis, but is evident in cortex and pith. As seen in cross section (Fig. 6 B), the outer region of the

cortex appears to become differentiated into two zones. The peripheral cells retain their rounded outline. Those adjacent become elongated radially. Strain develops which results in more or less appressed cells between the two zones (Fig. 6 C). Soon the lateral walls of the radially elongated cells present a shirred effect due to contraction along the lines of contact with neighboring cells. Obviously the total diameter of the root is now less than formerly. But if the strain is removed in part by a radial incision in the outermost zone of the cortex, the remaining tissue may be stretched to the wider diameter characteristic of an earlier stage. The two sets of contractions here considered in the tissues, are recorded in the whole root first by the longitudinal shortening which pulls the corm lower in the soil and second by the decrease in radial dimension of the older part of the root (Fig. 3 and 5).

*Cormel.* It is also in the region between the old and new corms that the buds giving rise to cormels are produced. These were relatively less numerous in the plants here grown, and their development was not studied inasmuch as detailed investigations have recently been made on these organs (6).

#### TIME RELATIONS IN DEVELOPMENT

As noted above, numerous mitotic figures were found in stem-tips and leaves in 12 to 18 days after planting. Undoubtedly earlier detailed study would have given some evidence of activity but adequate material was not available for determination of the time of the first visible indication of growth. Within 40 days the corms of the variety Halley had developed their first sheath leaves and in the most advanced plants the first of the foliage leaves had appeared. The height of the shoots then used for study varied from 7.5 to 11.5 cm. Microscopic study showed that the bud was already beginning to elongate at this time. Within another week when the shoot length was approximately 20 to 25 cm., flower primordia (2 to 4 rudiments) were found in sectioned material. The new corms at this time were about 0.7 to 0.8 cm. in diameter. In 60 days the shoot length was about 45 to 50 cm. and the inflorescence axis bore 7 to 10 primordia (Fig. 2). The new corm was increasing slowly in diameter and at this stage had begun to produce contractile roots in some cases. The erect axis was still very compact. By close observation it was possible to distinguish internodes as early as 52 days, but this became an easy matter in 70 days when the largest sample showed successive internodes from base upward, 1.3, 0.5, and 0.2 cm. in length and a flower spike 0.45 cm. long bearing 17 flower primordia. Externally this shoot was 73 cm. tall, showed three green foliage leaves, and bore eight contractile roots, of which the six oldest already showed wrinkles, due to contraction of the upper part.

Elongation continued, with the result that the strongest specimen,

about ten days later, was 88 cm. high, with four foliage leaves, an inflorescence axis over 4 cm. long with a spike of 17 flower primordia, and a good secondary spike (Pl. I, 12). Within another interval of ten days (90 days from planting) dissections showed that the flower buds had blasted. This was first evident on December 27, and showed in all subsequent samples of treated corms.

Inflorescence axes in the "blind" specimens dissected at 102 days varied in length from a fraction of a centimeter to 12 cm. in this set (Fig. 5); in other Halley material started August 28, a height of 21 cm. was attained before blasting occurred (observed on December 3). No external evidence indicated the loss of activity in the flower spike; the shoots of more than a meter in height presented four or five well-developed leaves and the new corm had grown to a diameter of about 2 cm. at this time.

All treated Halley plants not previously used were examined carefully on January 23, approximately 120 days after planting. In the field Halley plants require about 63 days for flowering as recorded at Amherst by Thayer (24). The interval here allowed would be adequate time for blooming if the plants in the greenhouse required twice the length of time they do in the field. On dissection young flower spikes were always in evidence varying in length, but always blasted (Fig. 5). This was uniformly the case in Halley except that one untreated plant remained plump and active, blooming on March 6, 119 days after the date of first activity noted in this lot. The spikes with as many as 17 primordia in some plants compared favorably with Halley in the field, described by Hottes (10) as free-blooming, with 13 flowers, and branching. In many cases the rudiments of axillary spikes were evident in preparations (Pl. I, 11). And in dissections it was obvious that more than one shoot per plant produced a spike as is characteristic of Halley in the field.

In this set there seemed to be no relation between the production of flower spikes and contractile roots. In some of these plants contractile roots were formed, in others not, although the strongest development in size and number of roots was apt to occur in plants showing the most advanced flower spikes. All corms bearing contractile roots showed that flowering spikes had been initiated, a point at variance with the results of Geiger (6) in the variety Topaz, where she found that forcing inhibited the production of cormels and contractile roots.

In the variety Alice Tiplady, development required a longer interval of time. While mitotic divisions were found at as early a date as in Halley, elongation was less rapid as seen both in shoot height and in the growth of the inflorescence axis within the bud. Consequently, the production of flower primordia began at a later date. The examination on November 30 showed some elongation but no papillae for flower development. The first flower primordia were seen in sections of buds killed on December 12 (75

days) when the shoots were 50 to 65 cm. tall and the first of the contractile roots were evident. In 90 days the spikes showed three to six rudiments. Elongation and differentiation went on gradually, with seven to nine primordia on inflorescence axes up to 2.5 cm. in length in 104 days, when the shoot was 70 to 85 cm. tall. These flowers were advanced enough to show good differentiation in the stamen set. Within less than three weeks, the axis had grown to about three times this length, with the spike proper 1 cm. long. The earliest spike came into bloom approximately the middle of February. Untreated corms which became active after the middle of December began to flower in late March, after treated corms had ceased blooming. Shoots which had not bloomed were dissected at this time; in the majority of these, whether main or side shoots, there were blasted spikes.

The length of time for production of differentiated floral tissue seems to be longer in both varieties here studied than that reported by Jones in Crimson Glow, a form requiring in the field about 80 to 84 days between planting and flowering according to Thayer (24). In histological study related to the formation of floral tissue Jones (12) found that "differentiation occurs and has become prominent at about five weeks from the time the corms are planted." Later (13) he again indicates that embryonic flowers of *Gladiolus* are formed a few weeks after the corms are planted.

It must be remembered that this material was grown in greenhouses at a very unfavorable time of the year. Alice Tiplady seems to have had some advantage in being a form which normally requires a longer time for flowering than Halley, in that 74 to 75 days represent the interval observed in the field (24). The greater delay in early differentiation threw Alice Tiplady into continuously more unfavorable conditions for growth. This brought about more delay in differentiation of flowers so that natural light was of longer duration and greater intensity by the time that flower stalks reached the stage of very rapid growth and elongation prior to blooming. Halley plants, on the other hand, approached this stage at the time of shortest days and least light intensity. Under such unfavorable conditions it has already been noted (19) that there is a tendency toward "blind" gladioli such as was seen in Halley. That the situation may be improved by supplementing natural daylight with artificial light has recently been stated by Jones (13) and by Weinard and Decker (27). The former investigator, using 100 watt lamps over plots of the variety Crimson Glow started in January, reports an increase of 63 per cent in the number of spikes and an average increase of 30 per cent in the number of flowers per spike. At the same time he states that the time interval from planting to flowering is increased by the use of artificial light in these experiments by approximately 15 days. Weinard and Decker report a production of more than twice as many flowers as in normal daylight, when the latter is supple-

mented by the use of 500 watt lamps from dusk to midnight, on corms of the variety Virginia planted on October 1, after early summer harvesting in California. Their results differ from those of Jones in that the blooms appeared ten days earlier than in the control.

#### SUMMARY

The life history of *Gladiolus* was studied with chief reference to corms of the varieties Halley and Alice Tiplady, with some material of three other varieties, treated in the fall of 1929.

The history of development in the varieties was found to be similar except for differences in time relations.

In the varieties chiefly used, mitotic divisions were numerous in 12 to 18 days after treatment, when there was external evidence of growth.

The structure of the leaf and contractile root was studied.

Development including lateral expansion of the basal internodes of the stem precedes elongation of the upper internodes with the production of a terminal flower spike.

Primordia first appear as papillae which differentiate into spathe and flower. The order in organogeny in the latter is stamen set, perianth, and carpel set.

The development of microsporangia and ovules with their respective spores is characteristic for monocotyledons. The embryo sac contains the egg, synergids with conspicuous filiform apparatus, polar nuclei, and large antipodal nuclei.

The fertilized egg produces a single row of cells developing a basal cell, short suspensor, and massive embryo. The root-tip, cotyledon, stem-tip, and first leaf are differentiated as the embryo elongates to almost the length of the seed.

The endosperm with thick hemicellulose walls develops by the usual stages of free nuclear division, parietal placing, and wall formation.

Data are given for the time required by the main varieties for the different stages described.

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## INFLUENCE OF PHOSPHORUS DEFICIENCY ON METABOLISM OF THE TOMATO (*LYCOPERSICON ESCULENTUM* MILL.)

SOPHIA H. ECKERSON

Several years ago Kraybill (7) found that when phosphate is limited nitrate accumulates in the tomato plant; that carbohydrates also accumulate, while the appearance in general is that of a low nitrogen plant. This indicates a breakdown of some step in the process of nitrate assimilation. In the attempt to find which stage is retarded or inhibited one naturally turns first to a study of nitrate reduction. Therefore, these experiments were planned to study the reductase activity together with any observable change in metabolism due to phosphorus deficiency.

Briefly, these studies have shown that there are two definite phases in the effect of phosphorus deficiency upon the metabolism of tomato plants: 1. There is a rapid decrease in reductase activity which results in nitrogen starvation. Although there is an abundance of nitrate present the plant can no longer use it. 2. After the soluble phosphorus compounds have disappeared the complex phosphatides begin to break down. This results in the disintegration of the protoplasm and therefore the disorganization of the cell.

### METHODS

Vigorous young tomato plants (variety Bonnie Best) carefully selected for uniformity, when about five inches high were transferred to sand and watered with solutions lacking phosphate. The control plants were given full nutrient solution. Approximately 100 plants were grown in each series. When the limit of fair condition of the phosphorus-deficient plants of a series was reached another series was started. Microchemical and anatomical studies were made of each series, and the reductase activity was determined at weekly intervals.

*Determination of reductase activity.* It was found by preliminary trials that two plants yielded sufficient juice to make duplicate determinations for two successive days; and that lots of two plants each from the same series and at the same stage of growth showed little variation. There was some variation in growth in each series. Usually two to five plants out of 50 grown without phosphate grew considerably more than the majority, and of the controls usually one or two grew less than the majority. The reductase activity of each of these was determined separately, but is not included in the tables.

The plants, collected between 9:00 and 10:00 in the morning, were separated into the desired portions which were kept turgid until used. The parts were ground separately in a mill and the juice squeezed out through

4-fold cheesecloth. The juice was then covered with a layer of toluene and kept in darkness at 10° C. At this temperature the reducase activity of tomato plant juice is maintained for five to fourteen days. The first set-up was usually made within six hours after extraction, the second within thirty hours.

*Set-up.* 1 cc. plant juice, 1 cc. 10 per cent  $\text{KNO}_3$ , and approximately 10 mgs. glucose were made up to 10 cc., put in a 50 cc. Erlenmeyer flask and brought to pH 7.2 to 7.4 with NaOH, using phenol red as indicator. Toluene (7 to 10 drops) was then added, the flask was shaken, and put in an oven at 35° C. After 17 hours the amount of nitrite was determined by means of the sulphanilic acid reagent. This reagent gives a clear deep pink to red color with nitrites. Sulphanilic reagent for nitrites (4, p. 136):

a. Sulphanilic acid	8.5 g.
Acetic acid (33 per cent)	150.0 cc.
b. Alpha-naphthylamine	0.1 g.
Distilled water	20.0 cc.
Acetic acid (33 per cent)	150.0 cc.

"Dissolve the alpha-naphthylamine by heating in 20 cc. of water, filter, then add the acetic acid. Combine solutions a and b and keep in a tightly-stoppered bottle." Standard nitrite solution (1, p. 229): "Dissolve 0.1097 g. dry C. P. silver nitrite in about 20 cc. of hot water, add 0.10 g. C. P. sodium chloride, shake until the silver chloride flocculates, and make up to 1 liter. Draw off 10 cc. of the clear solution and dilute to 1 liter. Each cc. of the last solution is equivalent to 0.0001 mg. of nitrogen as nitrite."

*Procedure.* Remove all the flasks from the oven. Shake each flask, draw out a 1 cc. sample, put it in a test tube, and dilute to 10 cc. with distilled water. Add 2 cc. of the sulphanilic reagent to each flask. If a yellow color develops instead of the clear red there is too much nitrite for the amount of the reagent. In such case take another 1 cc. sample, dilute to 50 or 100 cc. and mix thoroughly. To 10 cc. of this dilution add 2 cc. of the reagent. Put 10 cc. of the standard nitrite solution, equivalent to 0.001 mg. nitrogen as nitrite, in a test tube and add 2 cc. of the sulphanilic reagent. Allow about 20 minutes for development of the color. Then compare the depth of color of the samples from the reduction tests with that of the standard. A colorimeter having cups of 15 cc. capacity was used for all the comparisons in this work.

Duplicate nitrate reduction tests for each lot of juice were made on two successive days and, as an additional check, on different amounts of juice—usually  $1/2$ , 1, and  $1\ 1/2$  or 2 cc. If there is approximate agreement in the former and nearly proportional reduction in the latter case, one can be fairly confident that the results found are an expression of the ability of that juice to reduce nitrate to nitrite. The reduction of nitrate by different

concentrations of juice from tomato plants of three different series is given in Figure 1. In general the amount of reduction by either 1 or 2 cc. could be used as a measure of reducase activity. In the tables in this paper the amount of reduction by 1 cc. is given.

*Determination of acidity.* Determinations of the pH values of the juice of the tomato plants were made by means of the quinhydrone electrode. Observations were also made of the acidity of the different tissues by use of the Clark and Lubs (2, p. 65, 66) series of indicators directly upon sections.

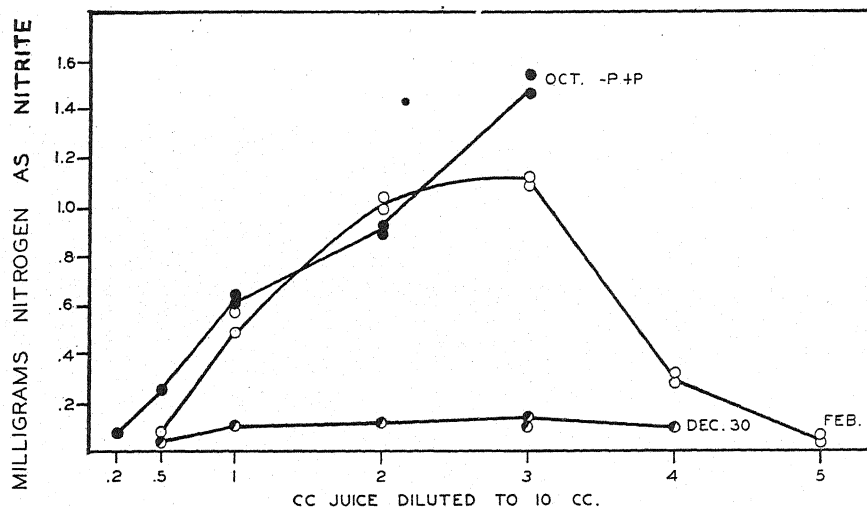


FIGURE 1. Reduction of nitrate; nitrite by different amounts of juice from three lots of tomato plants; showing that reduction by 1 cc. can be taken as a measure of reducase activity.

## RESULTS

### EXTERNAL CHARACTERISTICS OF PHOSPHORUS DEFICIENCY

The time required for external symptoms of phosphorus deficiency to appear depends, of course, upon the rate at which phosphate is built up into organic compounds and upon the amount present in the tissues at the beginning of the experiment. Starting with vigorous 4 1/2 to 5 inch plants containing abundant phosphate, under good greenhouse conditions in the spring and early autumn, definite symptoms appeared within two weeks. These are the purple midrib of the young leaves, and the smaller angle between petiole and stem. Usually by the end of the third week the plants had the form and appearance characteristic of shortage of phosphorus. The stems were short, stiff, and small in diameter. The upper leaves were small with purple veins. At this stage by their external appearance alone, it would be difficult to distinguish these plants from those deprived of ni-

trates. A little later, from the third to the fifth week, there are slight differences in aspect of the leaves. Those of the phosphorus-deficient plants become deeper green, the petioles retain their small angle with the stem while the midrib is curled downward and the leaflets inward, giving a plume-like appearance. In this they differ from the low-nitrate plants which become a paler green and do not curl. There is an increase in anthocyanin in the leaves of both series and a decrease in formation of glandular hairs. Figure 2 shows the similarity in appearance of the low-phosphate and the low-nitrate plants, as well as the slight difference in leaf aspect. These low-nitrate plants contained no nitrate but considerable phosphate whereas the low-phosphate plants contained an abundance of nitrate but only a trace of phosphate in the root tips.

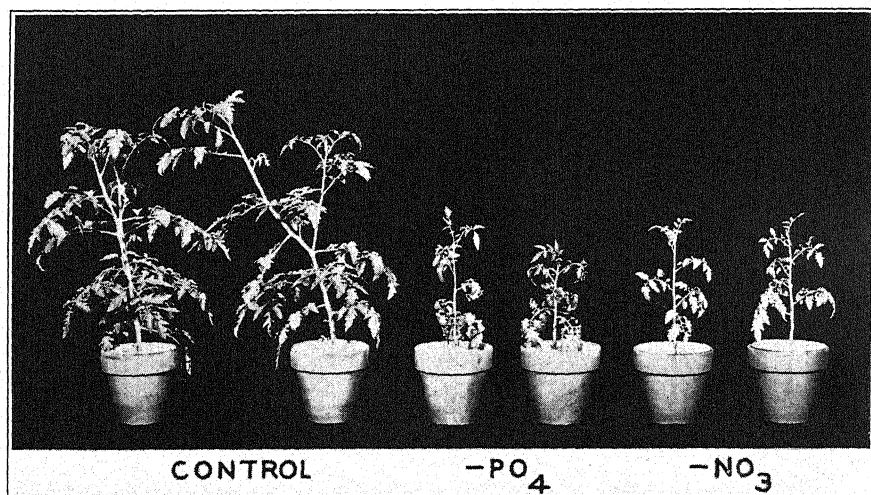


FIGURE 2. Phosphate-deficient compared with nitrate-deficient plants 4 weeks after transfer to sand; note similarity in amount of growth and general appearance; also the characteristic position and curling of leaves of the minus  $\text{PO}_4$  plants. *Lycopersicum esculentum*.

*Recovery of phosphorus-deficient plants when phosphate is supplied.* It was found from many experiments that, given favorable external conditions for growth, the rate and degree of recovery depends upon the extent of cell or tissue deterioration due to lack of phosphate. When definite external symptoms of deficiency first appear growth has almost ceased but there is no cell disintegration. There is still an occasional cell division at the extreme stem tip and in some of the root tips. If phosphate be supplied at this time, there is rapid resumption of growth and complete recovery. Under the conditions of these experiments when phosphate was withheld

until three to five weeks after the deficiency symptoms had appeared the plants did not recover. Usually there was only a feeble growth at the tip. Representatives of a typical series are shown in Figures 3 and 4.

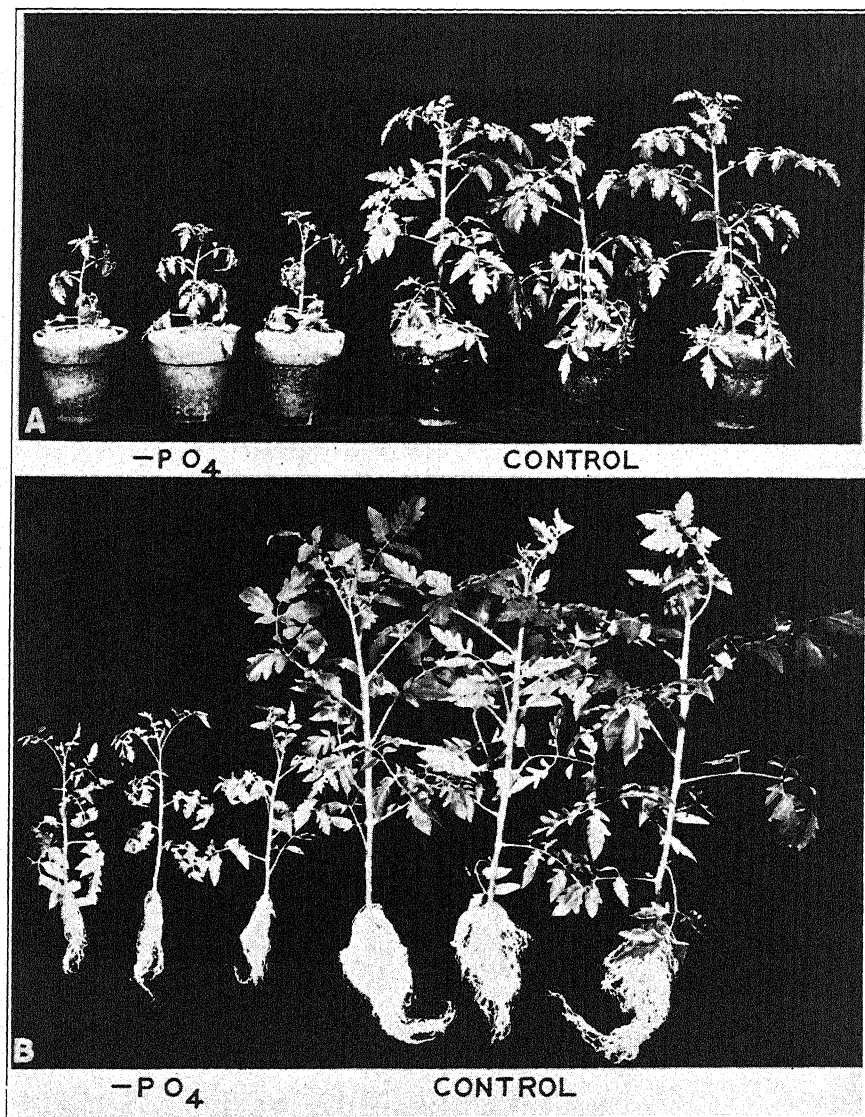


FIGURE 3. Phosphate-deficient compared with control plants 3½ weeks after transfer to sand; A, plants showing characteristic growth, and appearance of leaves; B, the roots of the same plants; note the white roots of the minus PO<sub>4</sub> plants as compared with their appearance 15 days later (Fig. 4).

This series was started August 23, 1928. About 3 1/2 weeks later (Sept. 17) the plants showed all the characteristic symptoms of phosphorus deficiency (Fig. 3 A). The roots were white and still in good condition, although growth had almost stopped (Fig. 3 B). The phosphorus-deficient plants were then separated into 7 similar lots of 12. Lot 7 was continued

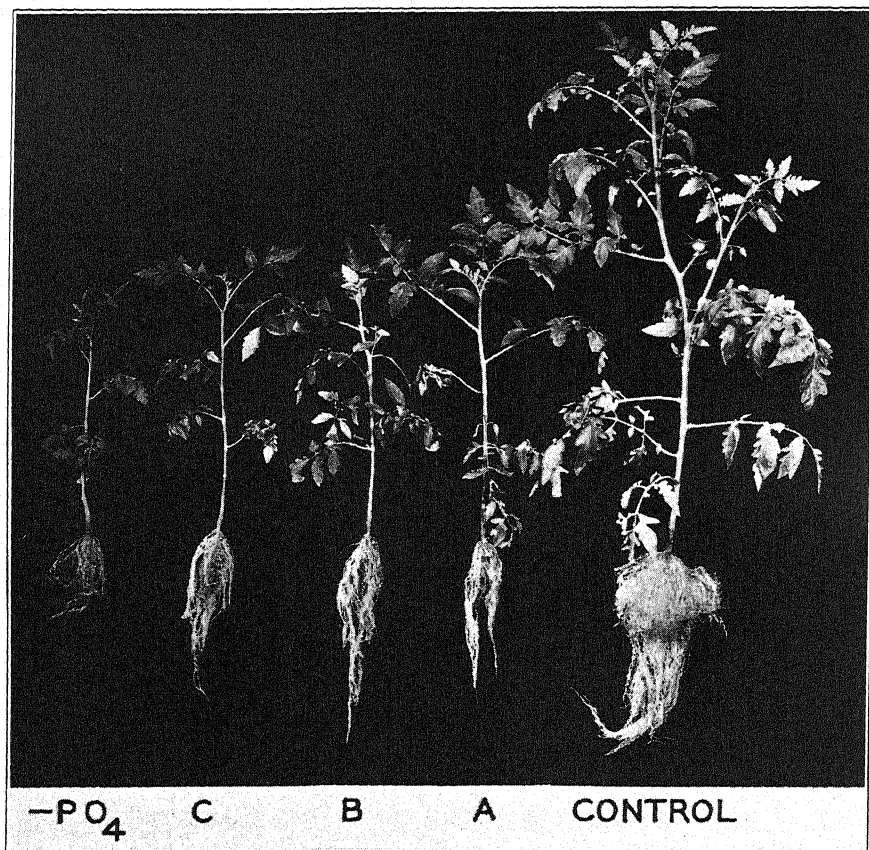


FIGURE 4. Effect of supplying phosphate at different stages of phosphorus deficiency; plants from same series as in Figure 3, 15 days later; plant A received phosphate in the early stage of phosphorus deficiency ( $-\text{PO}_4$ , Fig. 3) and shows 15 days' growth with phosphate; plant B received phosphate 5 days later and shows 10 days' growth with phosphate; plant C received phosphate 10 days later and shows 5 days' growth with phosphate; the next lot did not recover and plant minus  $\text{PO}_4$  shows the condition at the time phosphate was supplied; the light brown roots indicate the beginning of disintegration in the second phase of phosphorus deficiency.

without phosphate until the end of the experiment. Lots 1 to 6 were tested for recovery in succession at five-day intervals: Lot 1 being given phos-

phate at once (50 mgs.  $\text{KH}_2\text{PO}_4$  per plant), lot 2 five days later, lot 3 ten days later, and so on. Thus lot 6 was given phosphate on the twenty-fifth day after the plants were definitely deficient in phosphorus.

Lots 1, 2, and 3 made good growth and recovery. Lots 4 and 5 slowly developed one or two new leaves and a few new roots. Lot 6 did not recover.

The condition of lots 1, 2, and 3, 15, 10, and 5 days respectively after they had received phosphate is shown in Figure 4, A, B, and C. The first external effect upon supplying phosphate is a renewed growth of the roots and a rapid formation of new roots. Then the two upper leaves uncurl, the leaflets enlarge and soon a new leaf appears. Plant C, from lot 3 which had received phosphate five days before, shows these early stages of recovery. The poor condition of the phosphorus-deficient plants (Fig. 4,  $-\text{PO}_4$ ) accounts in part for the poor growth of lot 4 which was given phosphate at this time. It can be seen that the  $-\text{PO}_4$  plants had deteriorated considerably in the 15 days elapsed since lot 1 was started (Fig. 3). Growth at the tip had stopped, three or four of the lower leaves had dropped off and, most significant, the roots were in poor condition. Many were brown at the tips and some were beginning to disintegrate.

#### EFFECT OF LACK OF PHOSPHATE UPON NITRATE-REDUCING ACTIVITY

In every series after the plants were transplanted to sand and watered with nutrient solutions lacking phosphate, the inorganic phosphate within the plant decreased as growth continued, and at the same time the ability of the juice to reduce nitrates decreased. By the time the inorganic phosphate had been used up, with the exception of a trace at the tips of stem and roots, there was no reduction of nitrates by the juice, and there was a definite accumulation of nitrates within the tissues. When phosphate was

TABLE I  
EFFECT OF PHOSPHORUS DEFICIENCY UPON REDUCTION OF NITRATE BY TOMATO PLANT  
JUICE: REDUCTION BY 1 CC. JUICE (IN 1:10 DILUTION) IN 17 HRS. AT 35° C.

Series A 1929	Nitrogen (mg.) as nitrite			
	Tops		Roots	
	Phosphate added	Phosphate not added	Phosphate added	Phosphate not added
Aug. 13 Transferred to sand	0.71	0.71	0.91	0.91
Aug. 20	0.85	0.76	—	0.58
27	1.00	0.00*	0.79	0.02
Sept. 3	0.75	0.006	1.37	0.02
10	Lvs. 0.90    Stem —	Lvs. Trace    Stem 0.00	0.57	Trace

\* Plants just beginning to show external symptoms of phosphorus deficiency.



TABLE II  
EFFECT OF PHOSPHORUS DEFICIENCY UPON FRESH WEIGHT (GRAMS) AND ACIDITY (pH) OF TOMATO PLANTS

Series A 1929	Tops				Roots			
	Fresh weight (g.) 2 plants		pH of juice		Fresh weight (g.) 2 plants		pH of juice	
	Phosphate added	Phosphate not added	Phosphate added	Phosphate not added	Phosphate added	Phosphate not added	Phosphate added	Phosphate not added
Aug. 13	27.0	27.0	5.96	5.86	6.0	6.0	6.70	6.40
20	27.5	31.0			19.0	18.5		
27	53.0	45.0			30.0*	30.5*		
Sept. 3	84.0	73.5	Lvs. Stem 5.81 5.62	Lvs. Stem 5.69 5.43	43.0	42.0	6.43	6.01
10	Lvs. Stem 82.5 53.0	Lvs. Stem 46.0 35.0			51.0	47.0		

\* At this time the roots of the control plants (PO<sub>4</sub> added) were long and slender, while those grown without phosphate (PO<sub>4</sub> not added) were shorter and thicker.

again supplied to the plants, the ability of the juice to reduce nitrates was gradually regained, and the nitrates in the plants began to decrease.

*Loss of reductase by low-phosphate plants.* This is a representative series of control and low-phosphate plants in August and September when growth is rapid. Columns 2 and 4, Table I, show the reductase activity of juice from tops and roots of the control plants. Although there was some variation from week to week, reductase was high throughout the five weeks of the experiment. Columns 3 and 5 show the nitrate reduction by juice of the plants which received no phosphate after they were transplanted to sand on August 13. Two weeks later, August 27, when external symptoms of phosphorus deficiency were just beginning to appear, there was no nitrate reduction by top juice and but little, compared with the control, by root juice. At the end of the fourth week, September 10, there was only a trace of reductase left.

It is significant that the juice from low-phosphate plants had become extremely low in nitrate-reducing ability before external symptoms of phosphorus deficiency appeared. Also a comparison of the reductase activity given in Table I and the fresh weights given in Table II shows that it was only after the reductase had become very low that growth of the low-phosphate plants was definitely slowed.

The greater acidity of the juice from the low-phosphate plants shown in columns 5 and 9 of Table II is described in a separate section.

*Reductase regained when phosphate is supplied.* The plants were transplanted to sand September 5 and grown without additional phosphate for three weeks. The figures in Table III show that the second week (Septem-

TABLE III  
RENEWED REDUCTION OF NITRATE WHEN PHOSPHATE IS SUPPLIED TO PHOSPHORUS-DEFICIENT TOMATO PLANTS: REDUCTION BY 1 CC. JUICE (IN 1:10 DILUTION) IN 17 HRS. AT 35° C.

Series B 1929	Treatment	Nitrogen (mg.) as nitrite			Remarks
		Leaves	Stems	Roots	
Sept. 5	Transferred to sand; phosphate not added				
19		0.00	Trace	Trace	Plants showing first external symptoms of lack of $PO_4$
26	Photographed (see Fig. 5); then phosphate added	0.00	0.00	Trace	
30		0.01 Up- pers*	— Low- ers*	—	Growth of tip beginning
Oct. 18		0.00	0.05	Trace	
26		0.13	0.37	0.05	
31		0.02	0.60	0.37	

\* Uppers—Upper stems and leaves which developed after phosphate was supplied.

\* Lower—Lower stems and leaves.

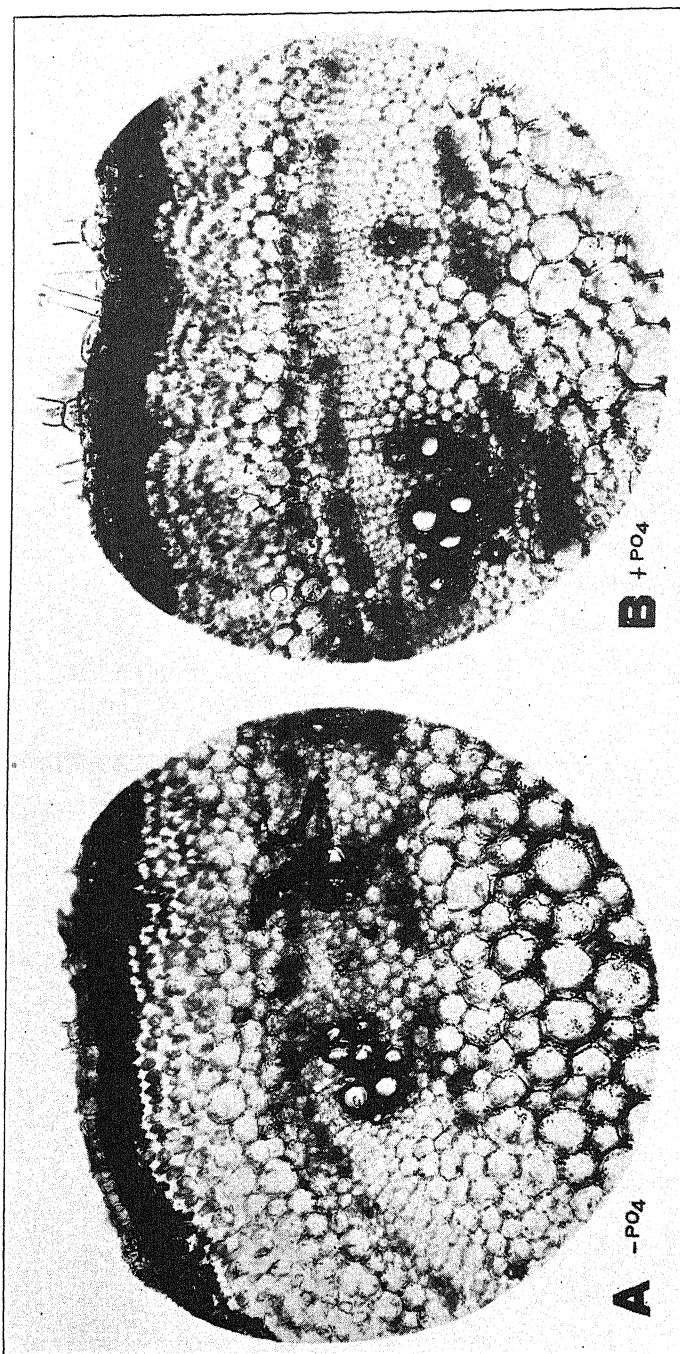


PLATE I. Stem structure of the minus  $PO_4$  and plus  $PO_4$  tomato plants shown in Figure 5. A, cross section from minus  $PO_4$  stem at the first node showing, compared with section B, narrower cortex, thicker walls, smaller internal phloem groups and much starch in pith. B, cross section from control (+ $PO_4$ ) stem; the chlorophyll-bearing cells in outer cortex are black in the photograph; there is starch in endodermis, little in other cells.

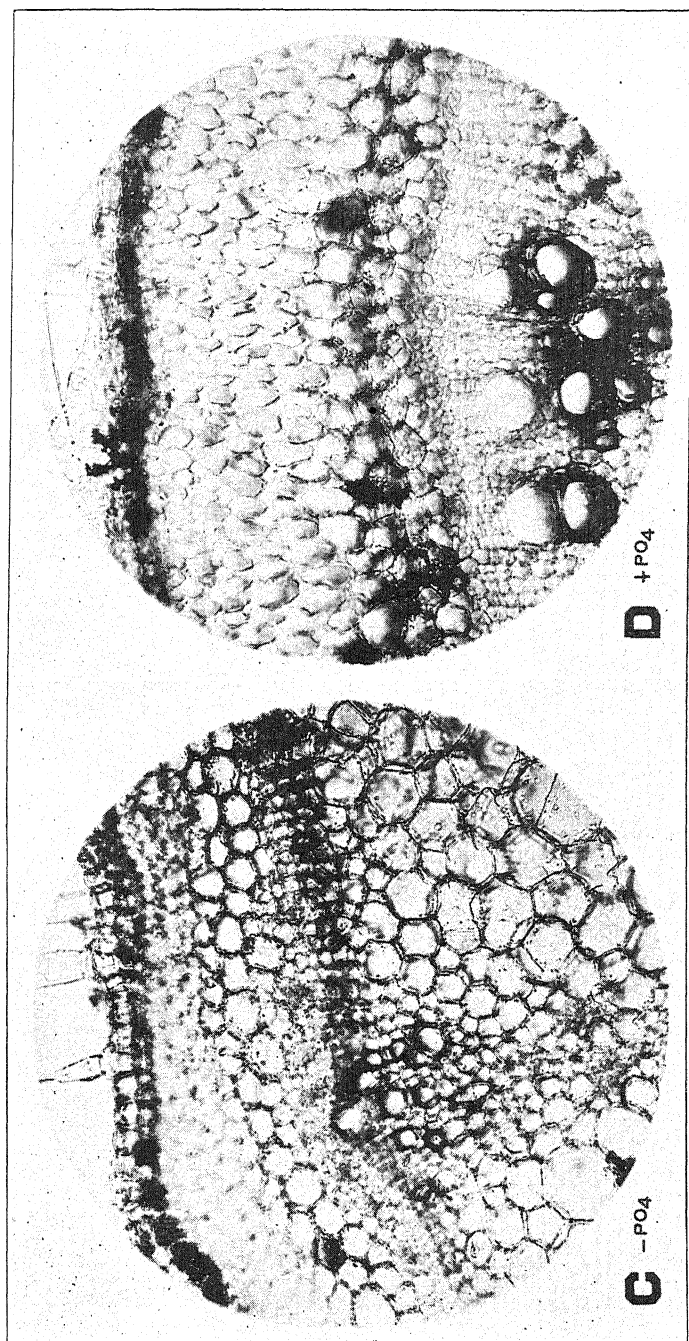


PLATE II. Stem structure of minus  $\text{PO}_4$  and plus  $\text{PO}_4$  tomato plants shown in Figure 5. C, cross section from minus  $\text{PO}_4$  stem at the third node showing, compared with section D, striking decrease of cambial activity; narrower cortex, smaller diameter of cortical cells, and xylem ducts; thickened walls of the xylem parenchyma cells and starch in the pith. D, cross section from control (+ $\text{PO}_4$ ) stem, showing high cambial activity.

ber 19), when the plants were beginning to show symptoms of lack of phosphate, they had lost nearly all ability to reduce nitrate. The next week, September 26, there was no reductase in leaves or stems and a mere trace in the roots. The plants now exhibited all the outward characteristics of phosphorus deficiency (Fig. 5, plants  $-PO_4$ ). The anatomical differences from the control plants are shown in Plates I and II, and described in another part of the paper.

Twenty of the plants were kept without phosphate and tested regularly.

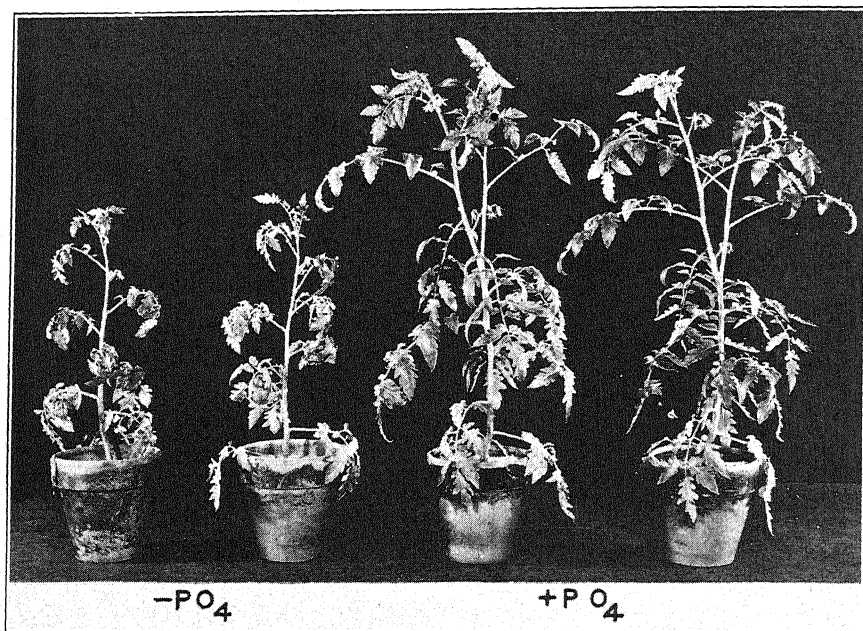


FIGURE 5. Phosphorus-deficient ( $-PO_4$ ) and control plants 3 weeks after transfer to sand; cross sections of the stems at the first and third nodes are shown in Plates I and II; Tables III and IV give reductase activity and acidity.

There was never any recurrence of reductase activity in the tops, and the roots never had more than a trace—far too little to measure. There continued to be an abundance of nitrate in the tissues.

The remaining 32 phosphorus-deficient plants were given 100 cc. each of 0.05 per cent  $KH_2PO_4$  on September 26 and October 3. September 30, four days after the first application, there was again some reductase in the leaves. This increased slowly at first and then more rapidly, especially in the roots, and older leaves and stems developed before phosphate was added. The accumulated nitrate and starch in the tissues decreased and

growth was fairly rapid. The figures for this regained reducase activity are given in Table III, beginning September 30.

The pH values of the juice from plants of this series given in Table IV are described in the section under "acidity."

TABLE IV  
EFFECT UPON pH VALUES WHEN PHOSPHATE IS SUPPLIED TO PHOSPHORUS-DEFICIENT TOMATO PLANTS: pH VALUES OF TOMATO PLANT JUICE

Series B	Leaves		Stems		Roots	
	Phosphorus deficient	Phosphate supplied	Phosphorus deficient	Phosphate supplied	Phosphorus deficient	Phosphate supplied
1929						
Sept. 19†	5.33*		5.50*		5.59*	
Sept. 26	5.08**		5.42**		5.42**	
KH <sub>2</sub> PO <sub>4</sub> supplied to 32 of the deficient plants	Uppers	Uppers*	Lowers	Lowers		
Oct. 18	5.33	5.52	5.13	5.50	6.18	5.60
26	4.91	5.75	5.38	5.67	5.67	5.84
31	5.08	5.75	5.28	5.45	5.38	5.89

† Sept. 19. The pH values of the control plant juice were: leaves 5.74, stems 5.67, roots 5.84.

\* New leaves somewhat smaller than control but still green.

\*\* Plants definitely phosphorus-deficient: leaves small, purplish, and petioles forming acute angles with the stem.

*Reducase lost when phosphate is low and regained when phosphate is again supplied.* The plants of this series were transferred to sand December 7. During December growth of the control plants was slow. After six weeks they were still only about one-half the size of spring or fall plants four weeks after transplanting. A comparison of the fresh weights given in columns 2 and 6, Table V, with fresh weights of an August series (Table II, columns 2 and 6), shows that the weekly increments were relatively low. Also the reducase activity was low—approximately one-tenth that of the August series.

The plants grown without additional phosphate were slow in exhausting the supply which they contained when transferred to sand. Reducase decreased slowly. A small amount of nitrate was being reduced during the first three to four weeks. There was some accumulation of starch but less than in the spring and fall series. Growth was somewhat retarded after the second week, as compared with the controls, but definite external characteristics of phosphorus deficiency did not develop until the fourth to the fifth week.

The figures in Table V show that by the second week reducase was definitely lower in the low-phosphate plants than in the controls. During the fourth week all reducase disappeared from the plant tops. The roots

TABLE V  
EFFECT OF PHOSPHORUS DEFICIENCY UPON REDUCTION OF NITRATE BY TOMATO PLANT JUICE:  
REDUCTION BY 1 CC. JUICE (IN 1:10 DILUTION) IN 17 HRS. AT 35° C.

Series D	Tops				Roots			
	Fresh weight (g.) 2 plants		Nitrogen (mg.) as nitrite		Fresh weight (g.) 2 plants		Nitrogen (mg.) as nitrite	
	Phosphate added	Phosphate* not added	Phosphate added	Phosphate* not added	Phosphate added	Phosphate* not added	Phosphate added	Phosphate* not added
1020 Dec. 7 Transferred to sand	32.0	27.0	0.0015	0.0007	9.0	4.5	0.0013	0.0008
	24.6	25.0	0.0000	0.0016	10.5	9.0	0.100	0.080
	42.0	20.5	0.050	0.007	15.0	11.5	0.037	0.003
	50.5	37.0	0.095	0.00	15.0	15.0	0.001	0.0007
1930 Jan. 2	—	40.0	—	0.00	—	15.0	—	0.0008
	46.5	30.5	0.070	0.00	12.0	15.0	0.10	0.0002

\* Jan. 4. Phosphate was supplied to some of the phosphorus-deficient-plants. Results shown in Table VI.

still retained a little. There was no recurrence of reducase in the tops until phosphate was again supplied.

TABLE VI

NITRATE-REDUCING ACTIVITY REGAINED WHEN PHOSPHATE IS SUPPLIED TO PHOSPHORUS-DEFICIENT TOMATO JUICE: REDUCTION BY 1 CC. JUICE (IN 1:10 DILUTION) IN 17 HRS. AT 35° C.

Series D	Nitrogen (mg.) as nitrite		
1930 Jan. 4 Phosphate supplied	Stems and Leaves		Roots
	Uppers*	Lowers	
Jan. 11	0.00	Trace	0.0008
16	0.028	0.0014	0.003
21	0.008	0.012	0.022
27	0.002	0.011	0.006

\* Growth after phosphate was supplied.

Thirty of the plants were given phosphate (50 mg.  $\text{KH}_2\text{PO}_4$  per plant) on January 4 and 16. The figures showing the gradual recovery of reducase are given in Table VI. Within 12 days after the first application of phosphate both tops and roots had regained a considerable amount of reducase.

#### ACIDITY

*Increase of acidity in phosphorus-deficient plants.* Without exception, as the plants became low in phosphate, their acidity increased. The pH values given in Table II show that for these plants the extracted juice from each organ—leaf, stem, and root—of the phosphorus-deficient plants was more acid than that from the corresponding organ of the plants supplied with phosphate (columns 4 and 8). This was true of leaves and stems in all the determinations made, and of roots for the first four or five weeks. But when phosphate was withheld until the roots began to darken and the tissues to disintegrate, it was sometimes found that the juice from such roots was considerably less acid than that from the control plants.

By means of the indicators it was found that the phloem, which in the control plants was slightly alkaline, becomes acid in reaction as soon as there is a deficiency of phosphate in the tissues. Next the acidity of the cambium increases. These early changes take place before any external indications of phosphorus deficiency are visible. Later the acidity of each tissue is greater than of the control plants.

*Decrease of acidity when phosphate is supplied to phosphorus-deficient plants.* The pH values of the extracted juice from plants of Series B are given in Table IV (see Table III for reducase). Columns 2, 4, and 6 show the acidity of the plants to which no phosphate was supplied. Columns 3, 5, and 7 show the decreased acidity after phosphate was added.



When the plants began to grow after phosphate was supplied, the tissues of the new growth had about the acidity of those of the control plants. They were definitely less acid than the older parts which had become phosphorus-deficient. However, within a month after the first application of phosphate (Sept. 26) the acidity of these older parts had decreased to about normal.

#### CHEMICAL AND ANATOMICAL CHANGES DUE TO PHOSPHORUS DEFICIENCY

*Phosphate; Nitrate.* When the plants were transferred to sand they contained sufficient phosphate for a week or more, depending upon the rate of growth. For the first few days it was abundant in all the tissues. Then it began to decrease in the older leaves and lower stem. Soon it was found only in the upper stem, the root tips, and in or near the cambium, with the exception of a little in the oldest leaves. Usually two of the basal leaves fell off about a week after transplanting and apparently these were never wholly depleted of phosphate.

At this time nitrate, which had been present in moderate amount, noticeably increased. Phosphate continued to decrease until at about the third week it could be found only in the extreme stem tip and in some of the root tips just back of the embryonic tissues. Nitrate increased until it was distributed in all the tissues in excessive amount.

*Potassium; Calcium.* Both potassium and calcium began to accumulate in the plants as growth slowed down. At the third or fourth week there was a great excess, especially in the upper and middle stems and leaves.

*Magnesium; Sulphate.* No definite increase of either magnesium or sulphate, with decreased growth of the plants, was noted. If there was any increase it was very slight.

*Sugars; Starch.* As soon as the nitrate-reducing activity decreased, sugars and starch began to increase, especially in the middle stems and leaves. At about the third week when all ability to reduce nitrate had been lost (appearance as in Fig. 5) starch had increased in the upper as well as the middle stems and leaves. Compared with the control plants, the phosphorus-deficient plants had much more starch per section across the stem at any level from the second node down to the fifth or sixth. The extreme tips of the phosphorus-deficient plants contained no starch and were similar in every way to the tips of the control plants. The basal stems of the deficient plants, like the control plants, contained abundant starch. There was no striking difference in amount. Later, the fifth to sixth week, the starch in the phosphorus-deficient plants began to break down and sugars, including sucrose, increased rapidly.

*Phosphatides; Proteins.* At the fifth or sixth week the protoplasm of many of the cells began to disintegrate. The phosphatides now reacted more readily, as though they had been freed from some combination or

organization. When sections of tissue were mounted in a few drops of distilled water, the phosphatides of the disintegrating cells formed a foamy, pseudo-cellular semi-solution. This does not occur when cells are in good condition, although such behavior upon the addition of a little water is characteristic for extracted phosphatides. Still later the phosphatides seemed to decrease in amount. This was shown qualitatively by the relatively smaller precipitate produced by cold acetone from alcohol-ether extracts, as well as by precipitation in the cells and reduction of osmic acid.

When disintegration of the cells began, the proteins first seemed to become more fluid. There was decreased precipitation by protein-precipitating agents, such as iodine potassium iodide solution, platinum chloride solution, and others. Next there was a breaking up of the proteins.

*Anatomy; Plates I and II.* During the second and third weeks as sugars and starch accumulate, the cell walls thicken. Collenchyma increases and the corner thickenings are thicker (by approximately 50 per cent) than in the control plants. Cambial activity in the middle and lower stem decreases and soon ceases. Calcium oxalate crystals accumulate in the phloem parenchyma cells. The xylem parenchyma cell walls thicken and become lignified.

Plates I and II show cross sections of the stems of the plants in Figure 5. The phosphorus-deficient ( $-PO_4$ ) plants had lost nearly all reducase activity (Table III) and sugars and starch had accumulated. The anatomical features of the extreme stem tips of the  $-PO_4$  plants were like those of the control plants, but just back of the tip at the second node there were some changes (Pl. I A,  $-PO_4$ ; B,  $+PO_4$ ). The collenchyma cell wall thickenings were heavier, and there was somewhat less external and considerably less internal phloem than in the control plants. Also there was much more starch in the pith cells. At the third node all of the characteristic changes appear in some degree (Pl. II C,  $-PO_4$ ; D,  $+PO_4$ ). The most significant change was the absence of new secondary xylem and phloem cells, owing to loss of cambial activity. The cambium of the control plants was highly active in this region. Photograph D shows a layer of xylem mother cells and phloem mother cells on either side, together with much new thin-walled xylem and phloem parenchyma. Photograph C shows no such new elements; moreover the older xylem parenchyma instead of being thin-walled is thick-walled and lignified. In the photograph the walls of these two to three layers appear dark because they were stained with thionin. Other changes in the  $-PO_4$  plants were a narrow cortex and small pith containing much starch. The cells of each tissue were smaller in diameter with thicker walls than of the corresponding tissues in the control plants.

At lower levels down to near the base of the stem these differences between the  $-PO_4$  and  $+PO_4$  plants were intensified. There was less difference at the base.

## DISCUSSION

The tomato plant when grown without phosphate behaves for the first three to four weeks as though grown without nitrate. Several workers have noted the similarity in external appearance. This similarity is true also for the internal characters. The anatomical characters observed in these series of phosphorus-deficient plants match those described by Kraus (6, p. 71-79) for nitrate-deficient plants. A little later, however, there is a difference. The no-nitrate plant continues to store starch and cell wall substances for many weeks, while in the no-phosphate plant starch storage stops at about the fifth week and starch previously stored breaks down. Kraybill (7) noted this difference. He states: "The plants resemble the nitrogen-deficient plants in form and show an increase in free reducing sugars, sucrose, and starch. Starch, however, does not continue to accumulate as in the case of the nitrogen-deficient plants, although free reducing sugars and sucrose do in large amounts."

MacGillivray (8) noted the later decrease in starch but not the early increase. He analyzed tomato plants after 52 and 63 days' growth without phosphate. That the plants were in a state of acute phosphorus starvation is evident from his statement (p. 108) that "the experiment" (F, 63 days) "was considered complete when two of the no-phosphorus plants had died"; also from his description of the roots of both series (p. 108, 104): "The roots of the no-phosphorus plants were . . . dark brown in color, especially at the ends. . . ." MacGillivray found that "the quantity of reducing sugars and the total quantity of sugars increased greatly in the plants grown in the absence of phosphorus as compared with the quantities in . . . the control plants," and that "These increases in sugars were usually accompanied by a lower percentage of starch."

In regard to the forms of nitrogen in the no-phosphorus plants as compared with the control plants, MacGillivray found "a decrease in percentage and also total amount of coagulable nitrogen and an increase in water-soluble forms, excluding nitrates." Kraybill (7) found that the increase in water-soluble forms of nitrogen included, in stems of low-phosphorus plants, increases in ammonia, nitrate,  $\alpha$ -amino acid, and amide nitrogen. The amount of these forms of nitrogen was four times that in the plants grown in complete nutrient solutions.

There are two possible explanations of this increase in water-soluble forms of nitrogen in the phosphorus-starved plants. Amino acids and amides may have been synthesized in the early stage of phosphate deficiency while there was still some reduction of nitrate, but when perhaps the conditions necessary for further synthesis into proteins may have been lacking; or they may have come from autolysis of proteins in the late stage of phosphorus starvation.

It is interesting that, after all the phosphate in the tissues has been used up, the first cell substances to break down are those containing combined phosphate, i.e., starch (12, 13, p. 63) and lecithin. The starch breaks down first, and after most of it has disappeared the phosphatides begin to break down. Although it is not certain that this phosphorus can be re-utilized, it seems possible. Koch and Reed (5) have shown that when *Aspergillus niger* is grown in solutions containing an insufficient amount of phosphorus the water-soluble forms of inorganic and organic phosphorus are used up first. Then the lecithin phosphorus decreases, but the nucleo-protein phosphorus decreases only slightly if at all. The authors think that of these three groups of phosphorus compounds, the nucleo-proteins are of the greatest importance to the life of the cell; lecithin comes next in importance, although some of its phosphorus may be re-utilized; and "the water-soluble forms are the ones from which all others are built up." Reed (10, p. 522-526) has studied the effect of lack of phosphorus directly upon cells of *Spirogyra* and other Algae. He states that "cells in a state of phosphorus starvation first lose the soluble phosphorus complexes; later the injury extends to the strictly living organs of the cell, finally resulting in its death." That expresses the relation of the two phases of injury in the tomato. Reducase activity decreases with the decrease in soluble phosphorus compounds until when all the phosphate is lost all reducase activity is lost. The plant then gradually assumes the appearance and character of a plant grown without nitrate. Injury is not severe at this stage and the plant recovers readily upon supplying phosphate.

Starch storage stops at this stage. If amylophosphoric acid is an essential constituent of the starch grain (11, p. 51-61) the lack of phosphate would prevent starch formation just as it prevents further synthesis of phosphatides and nucleo-proteins. Then these phosphorus-containing substances in the cells begin to break down in the order: starch, phosphatides, proteins. This is the beginning of the second phase, in which the injury becomes fatal. Soon after the phosphatides begin to break down, the protoplasm is disrupted and the whole cell is disorganized. Even at a stage when the proportion of disorganized cells is still small, the addition of phosphate is of little avail. The plant does not recover.

The early high-carbohydrate, nitrogen-starved (with much nitrate present) phase is due primarily to loss of reducase activity. It is not specific for phosphorus deficiency except in so far as the presence of phosphate in the tissues is one of the essential conditions for reducase activity. The presence of potassium also seems to be necessary. Nightingale, Schermerhorn, and Robbins (9) found that in tomato plants grown with abundant phosphate but without potassium, nitrate accumulated. At the end of three to four weeks, there was "a heavy deposition of starch in the parenchymatous cells of the cortex, phloem, and medullary rays" and "the pith cells were

packed with starch grains." "The anatomical structure of the minus-potassium stems was apparently identical in every respect with that of a typical nitrogen-starved, high carbohydrate tomato plant as described by Kraus and Kraybill," (9, p. 7). The reducase activity of minus-potassium plants supplied by Nightingale (9, p. 33) was found to be extremely low. Here, apparently, as in the phosphorus-deficient plants the early heavy storage of carbohydrates is due to the inability of the plants to utilize the nitrate. In both cases the later effect seems to be specific for the particular substance studied. The Biloxi soybean when subjected to an 8-hour photoperiod makes little growth. The plants have short stiff stems which contain much nitrate and starch (9, p. 4). Such plants were extremely low in reducase activity.

On the other hand, plants grown without nitrate were higher in reducase activity than the control plants (3). When nitrate was supplied to nitrate-deficient plants, nitrate reduction, hydrolysis of starch, and synthesis of amino acids began at once at a rapid rate. When phosphate was supplied to phosphorus-deficient plants at the early high-starch stage, such as those shown in Figure 3 or Figure 5, it was found in all parts of the plant within 30 minutes (in some cases 15 minutes) after it had been added to the pots. Yet an appreciable time elapsed before reduction of nitrate was observed; and hydrolysis of starch and synthesis of amino acids were slow at first. It seems as though some time is required to synthesize reducase or perhaps to activate some substance already present.

#### SUMMARY

1. Plants grown with abundant nitrate but without phosphate are, in about three weeks, essentially nitrogen-starved plants; they have the internal characters and the external appearance of plants grown without nitrate.

2. Reducase activity decreases as the phosphate and water-soluble organic phosphorus compounds decrease; when they are used up there is no further reducase activity.

3. A series of related changes follows the loss of reducase activity; nitrate accumulates, sugars and starch increase, acidity increases, cell walls thicken, cell division at root tips and stem tip decreases, and cambial activity ceases.

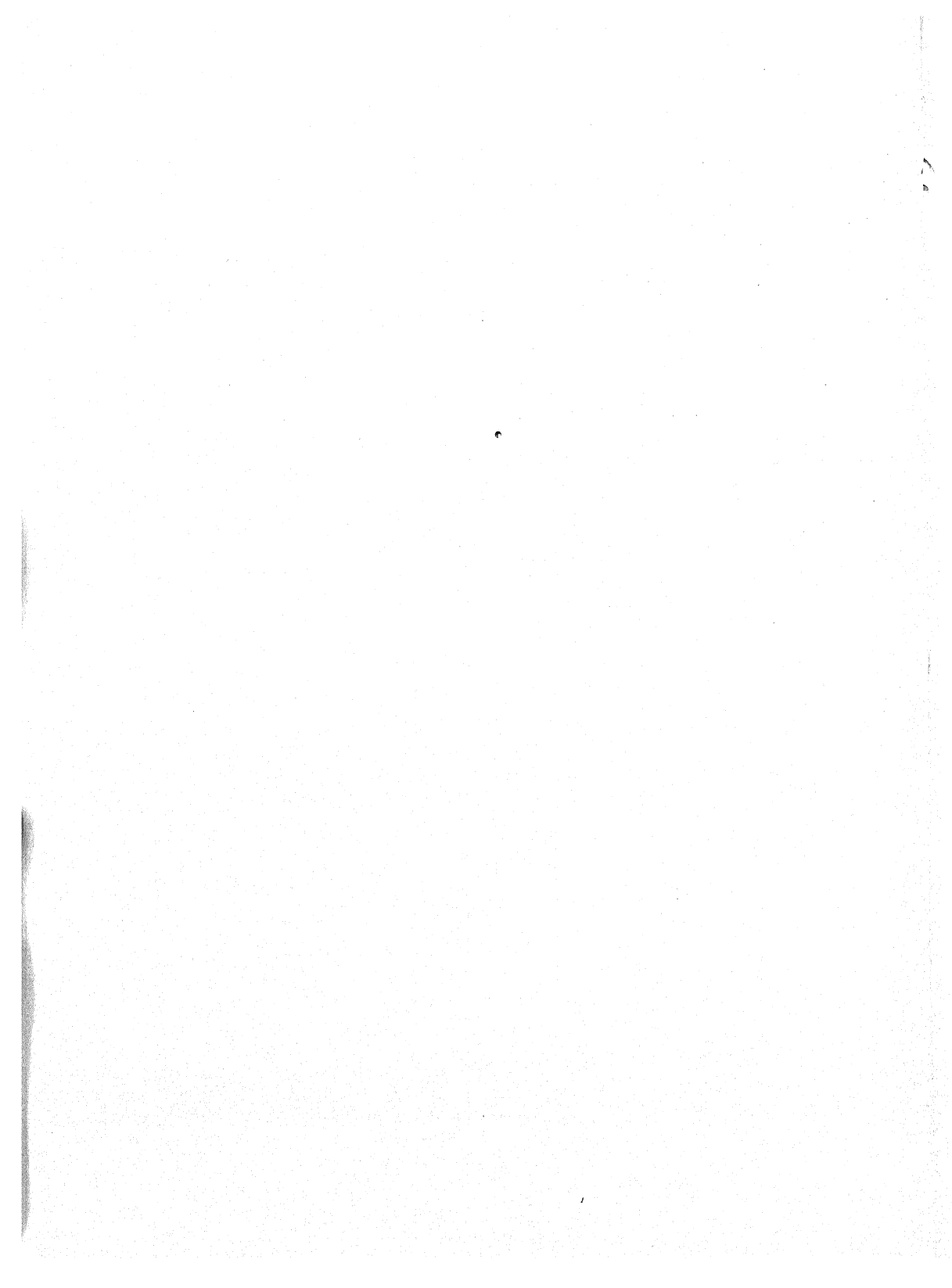
4. At about the fifth week phosphorus starvation becomes acute and the complex phosphorus compounds break down; starch decreases rapidly, and phosphatides and proteins begin to break down; the plant does not survive very long after this.

5. When phosphate is supplied to phosphorus-deficient plants at the early high-starch stage, reducase activity is slowly regained; nitrate and starch decrease slowly at first, then more rapidly; acidity decreases; finally

there is renewed cambial activity at the stem tip and renewed root development.

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# THE EFFECT OF CARBON DIOXIDE ON FRUITS AND VEGETABLES IN STORAGE

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## INTRODUCTION

Unusual variations in the concentrations of carbon dioxide and oxygen in the atmosphere around stored fruits and vegetables brought about either by respiration or artificial means may delay senescence or have an injurious effect on the tissues. Published data show that such effects are influenced by temperature, duration of storage, and variety of material under observation.

Reports of the Great Britain\*Food Investigation Board (10) show that the storage life of apples, plums, and strawberries may be prolonged by carbon dioxide storage. The carbon dioxide was produced by respiration of the fruit in air-tight storage rooms where ventilation was used to maintain the concentration of carbon dioxide within the range of 0 to 15 per cent and to prevent the concentration of oxygen from falling below 5 per cent. Kidd (13) and Kidd and West (15) reported that under gas storage conditions of 12 per cent CO<sub>2</sub> with 9 per cent O<sub>2</sub> at 8.1° C. and 9.2 per cent CO<sub>2</sub> with 11.8 per cent O<sub>2</sub> at 6.8° C., it appeared that the rate of respiration of apples was approximately halved and the length of storage life approximately doubled. Kidd and West (17) and Kidd, West, and Kidd (18) have shown that carbon dioxide storage (10 per cent CO<sub>2</sub> with 11 per cent O<sub>2</sub> at 8.1° C.) retarded the ripening of apples, preserved the green, firm, juicy condition, and extended the storage life approximately 1.5 times that of the controls for three varieties tested. Carbon dioxide storage at 8.1° C. and cold storage at 1.1° C. were equally effective in prolonging the storage life of apples. An extended period of exposure to excess carbon dioxide (above 15 per cent) or to an atmosphere containing no oxygen resulted in premature death of the fruit or the development of "brown heart."

Brooks, Cooley, and Fisher (5, 6) noted that although the flavor of Grimes Golden apples was changed by treatment with 100 per cent carbon dioxide for two days at 30° C., the fruit was not altered by a like concentration when held at 10° C. for six days. After both treatments the apples developed color in storage much more slowly than the untreated fruit. Brooks and Cooley (4) found that apples stored in concentrations of carbon dioxide greater than 5 per cent at 15° C. for five to seven weeks developed a pungent alcoholic flavor besides remaining green, firm, and crisp for a longer period than the untreated fruit after removal to warm air. In

<sup>1</sup> The writer is greatly indebted to the Dry Ice Corporation of America for furnishing funds and for making helpful suggestions in carrying on this investigation.



air-tight containers the apples produced more than 50 per cent carbon dioxide, developed a strong odor of alcohol, were nauseating to the taste, and soon became brown and soft. Magness and Diehl (20) showed that apples held for ten days at 22° C. in various percentages of carbon dioxide with 20 per cent oxygen did not soften as rapidly as the controls. They report that 5 to 10 per cent carbon dioxide caused no change in flavor, 20 per cent only a slight change, but in 50 per cent carbon dioxide the fruit was entirely inedible.

Thatcher (26) observed that raspberries, blackberries, and loganberries held in carbon dioxide remained firm for seven to ten days as compared to three days for the controls. He further suggested that the accumulation of carbon dioxide as one of the end products of enzymatic activity would bring about a retardation in the breakdown of apples during storage.

Hill (11) suggested that carbon dioxide must decrease hydrolysis of pectose since peaches in the gas did not soften or change in color during four weeks storage. When removed from storage the peach flavor was bitter and nauseating, and the fruit browned very rapidly.

Investigators have prolonged the storage life of pears as much as 50 per cent by storage in nitrogen (16), or in nitrogen with 10 per cent or less carbon dioxide and no oxygen (28). Trout (28) further showed that higher concentrations of carbon dioxide brought about pear core breakdown.

Barker (1) noted that 28 to 30 per cent carbon dioxide and 4 to 6 per cent oxygen in storage atmospheres produced in oranges a bad flavor, a bad odor, and a white crystalline precipitate on the carpel walls. Onslow and Barker (23) showed by analyses that the juice of oranges injured by carbon dioxide storage had a much higher alcoholic content than that of the controls.

Gore (9) and Lloyd (19) reported that carbon dioxide is effective in rendering hard persimmons non-astringent without causing softening or injury to the fruit if processed correctly. Gore also discussed over-processing of the fruit which resulted in darkening of the color during softening which, as he stated, was probably due to death of the tissues.

Overholser (24, p. 551) was able to store Fuerte Avocados in 4 to 5 per cent CO<sub>2</sub> with 4 to 5 per cent O<sub>2</sub> at 7.2° C. one month longer than the control. He found that 20 to 25 per cent carbon dioxide prevented softening of the tissue so that it remained tough after removal from the storage. This concentration of carbon dioxide did not bring about an objectionable flavor during or after storage.

Nelson (22) investigated some non-parasitic diseases of crucifers, lettuce, and potatoes in storage where he found spotting or discoloration of the tissues due to inadequate supply of oxygen. He found that temperature, when so low as to prevent utilization of the oxygen present, was an important factor in the production of the injuries. The accumulation of

carbon dioxide, however, was not found to be a factor in the production of the injuries studied.

Kidd (12) found that in potato storage 10 per cent carbon dioxide reduced sprouting, while 20 to 40 per cent inhibited sprouting and caused some injury to the tubers. Bartholomew (2), Stewart and Mix (25), and Bennett and Bartholomew (3) have found that blackheart of potato tubers results from a deficiency of oxygen in the storage chamber and not to the accumulation of carbon dioxide.

In a recent paper, the writer (27) has shown that 15 per cent carbon dioxide is effective in prolonging the life of cut flowers by retarding the opening of the buds while in storage.

This paper reports experiments dealing with the effect of carbon dioxide on fruits and vegetables held in storage at various temperatures. The object of the investigation was to determine the range of concentrations of carbon dioxide that would not injure the plant tissue during storage. The data presented show that the concentrations of carbon dioxide causing injury were not uniform for the fruits and vegetables tested. No attempt was made at this time to determine the critical concentration of carbon dioxide for each fruit or vegetable. The results as reported are applicable only to short periods of storage of fruits and vegetables.

#### MATERIAL AND METHODS

Twenty-two kinds of fruits and vegetables of commercial importance were used in this investigation (Table I). The citrus fruits were obtained directly from a citrus grower in Florida. The bananas were obtained from the United Fruit Company steamers as soon as they arrived in New York City. All other fruits and vegetables were procured from a local wholesale dealer who cooperated in furnishing only the freshest and best plant material. Approximately 4.4 liters (dry measure) of the fruits or vegetables were placed in each storage can. However, this quantity was varied somewhat depending upon the material tested, for example, either three to five stalks of celery, two bunches of asparagus, two to three heads of lettuce, or ten fingers of bananas.

During treatment the fruits and vegetables were placed in 35-liter tin cans (size 31.12 by 45.72 cm.). The desired concentration of carbon dioxide was obtained by placing a weighed amount of commercially manufactured "Dry Ice" (solid carbon dioxide) in a dish suspended inside of the can 2.54 cm. from the top. The weight of "Dry Ice" used (6.5 gms. for 10 per cent, 23 gms. for 25 per cent, and 50 gms. for 50 per cent) was not sufficient to alter greatly the temperature in the can for any length of time or to freeze the plant tissue. Gas analyses for the determination of the carbon dioxide content of the atmosphere in the cans were made during the first day and again at the end of the test period. The concentrations (per cent by vol-

TABLE I

KIND AND VARIETY OF FRUITS AND VEGETABLES HELD IN CARBON DIOXIDE STORAGE AND THE PERIOD OF THE YEAR THAT THEY WERE TESTED

Material	Period tested month—1929
Apple ( <i>Pyrus malus</i> L.)	August
Dutchess	Aug.-Sept.
Gravenstein	Jan.-Mar., Oct.-Nov.
Delicious	Feb., Oct.-Nov.
McIntosh	
Asparagus ( <i>Asparagus officinalis</i> L.)	May-June
Pedigree Washington	Feb.-May
Banana ( <i>Musa sapientum</i> L.)	
Bean ( <i>Phaseolus vulgaris</i> L.)	May-June
Slat Stringless	
Carrot ( <i>Daucus carota</i> L.)	June
Oxheart	June
Cauliflower ( <i>Brassica oleracea</i> L. var. <i>botrytis</i> D.C.)	
Celery ( <i>Apium graveolens</i> L.)	Jan.-Mar.
Golden Self-blanching	
Grapefruit ( <i>Citrus grandis</i> Osbeck)	Feb.-Apr.
Walters	April
Foster Pink	April
Thompson Seedless	April
King orange ( <i>Citrus nobilis</i> Lour)	Jan.-Mar.
Lettuce ( <i>Lactuca sativa</i> L.)	Feb.-Apr.
Mushroom ( <i>Agaricus campestris</i> L.)	Feb.-Apr.
Sweet orange ( <i>Citrus sinensis</i> Osbeck)	Apr.-May
Pea ( <i>Pisum sativum</i> L.)	
Peach ( <i>Prunus persica</i> Sieb. & Zucc.)	Aug.-Sept.
Georgia Belle	
Pear ( <i>Pyrus communis</i> L.)	Jan.-Mar.
Anjou	Aug.-Sept.
Bartlett	
Potato ( <i>Solanum tuberosum</i> L. K.)	July-Oct.
Irish Cobbler	June
Radish ( <i>Raphanus sativus</i> L.)	
Rhubarb ( <i>Rheum rhaponticum</i> L.)	June
Victoria	Jan., Mar., and June
Spinach ( <i>Spinacia oleracea</i> L.)	Apr.-June
Strawberry ( <i>Fragaria chiloensis</i> Duch.)	April
Tangerine ( <i>Citrus nobilis</i> var. <i>deliciosa</i> Swingle)	
Tomato ( <i>Lycopersicum esculentum</i> Mill.)	Aug.-Sept.
Greater Baltimore	

ume) of carbon dioxide obtained by the above method resulted in a decrease in the oxygen content of the container. Data taken from a series of experiments where carbon dioxide percentages of 25, 50, 67, and 81 were obtained showed 14, 9, 6, and 4.5 per cent oxygen respectively at the beginning of the test period. The carbon dioxide was confined in the cans by applying paraffin to the seams and plasteline clay to the edges of the tops. Each can top was sealed on at the beginning of the experiment and again after gaseous equilibrium was established between the atmosphere of the can and that of the temperature control room.

The fruits and vegetables were held in carbon dioxide storage at various

temperatures ( $0^{\circ}$ ,  $4^{\circ}$ ,  $10^{\circ}$ ,  $15^{\circ}$ ,  $21^{\circ}$ , and  $25^{\circ}$  C.) for a period of time approximating that required for shipment from the producer to the market. For this reason the period of treatment was varied from three to seven days for most of the fruits or vegetables. Potatoes, however, were held in carbon dioxide storage for as long as 34 days to observe the rate of sprouting or the resulting injuries. The tests were replicated once with potatoes and from two to seven times depending upon the nature of the other plant material.

Fruits and vegetables treated with carbon dioxide were compared with two sets of controls, one that had been held in air in the room, and one that had been sealed in the can without added carbon dioxide. At the end of the storage period the control and treated fruits and vegetables were held together in air at  $21^{\circ}$  to  $25^{\circ}$  C. to note external and internal appearances of color, odor, and taste. These observations were recorded immediately after the plant tissue was removed from treatment and daily thereafter for a period of 1 to 14 days while in warm air.

No attempt was made to control the relative humidity of the air in the test cans during the period of treatment. However, a number of tests were made to determine the injurious effects of the carbon dioxide on wet and relatively dry surfaces of fruits and vegetables. In most cases the relative humidity of the air in these cans was very high. This is especially true of the tests where rapidly respiring plant tissue of high moisture content was held at the higher temperatures. At the termination of the storage period for some fruits or vegetables, droplets of moisture were found on the walls of the can, but in most tests no excess moisture was present.

## EXPERIMENTAL RESULTS

### EFFECT OF CARBON DIOXIDE ON FRUITS

#### *Apple*

The extent of the noticeable injury to apples stored in carbon dioxide at different temperatures varied with the variety according to the firmness of the fruit. Data in Table II show that the Dutchess, a soft, cooking apple, was injured by 50 per cent carbon dioxide while the Delicious, a relatively hard, eating apple, was not injured by 83 per cent carbon dioxide when held in storage for seven days at  $0^{\circ}$  C. The noticeable changes in the apples exposed to high concentrations of carbon dioxide were as follows: the development of an abnormal flat, somewhat bitter flavor in the harder apples (Delicious and Gravenstein); a noticeable fermentative or alcoholic flavor for the softer apples (Dutchess and McIntosh); and a pronounced internal browning of the tissue about the core and just under the skin as shown in Figure 1. This internal browning referred to as "brown-heart" by Kidd and West (14) was much more pronounced in the soft than in the firm apples. It appears in Figure 1, that the Dutchess apples treated with

TABLE II

EFFECT OF VARIOUS CONCENTRATIONS OF CARBON DIOXIDE ON FRUITS STORED  
AT DIFFERENT TEMPERATURES

Material	Days of storage	Per cent CO <sub>2</sub> at which no injury was observed at various temperatures in ° C.						Per cent CO <sub>2</sub> at which injury was observed at various temperatures in ° C.					
		0	4	10	15	21	25	0	4	10	15	21	25
Apple													
Dutchess	6-7	25	25	*			*	50	50	28			25
Gravenstein	7	46	30	*			*	64	50	27			25
Delicious	7	83	67	50			*	78	70				26
McIntosh	6-7	48	25	25			25	65	50	50			50
Banana	7			*	33	33				25	40	40	
Peach													
Georgia Belle	4	20	20	10			*	30	30	20			10
Pear													
Anjou	7	42	25		*			62	40		30		
Bartlett	7	10	10	10			*	28	28	28			12
Strawberry	3	15	15	15				25	25	25			

\* Concentration of carbon dioxide not determined.

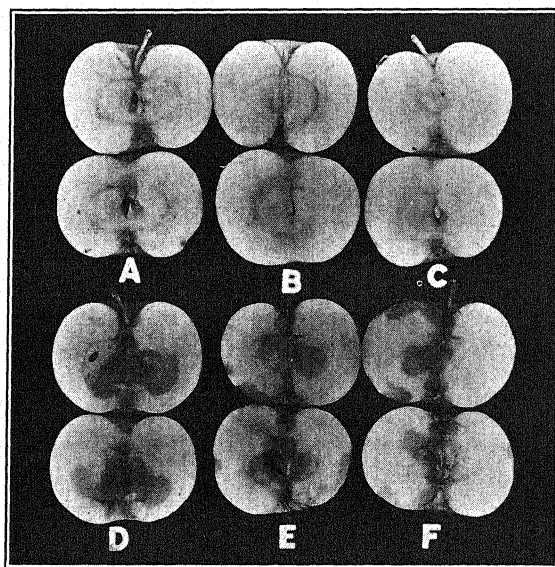


FIGURE 1. Dutchess apples held in carbon dioxide for six days at 4° C. A. Control in room, B. control in can, C. 25 per cent, D. 50 per cent, E. 70 per cent, and F. 87 per cent carbon dioxide.

50 per cent carbon dioxide were injured to a greater extent than those in higher concentrations. This, however, was not the case since the higher concentrations caused browning throughout, much of the discoloration occurring just under the skin. As the storage temperature was raised, the carbon dioxide injury was intensified so that at 25° C. the treated fruit often broke down completely during storage. All apples not injured by the carbon dioxide treatment were held in air from seven to ten days at 21° to 25° C. without showing detrimental effects.

Delicious apples that had been held in normal cold storage from the time of harvesting until February were placed in various concentrations of carbon dioxide at 4° C. for one week. When placed in carbon dioxide storage the fruit was firm and somewhat juicy, but when removed, the apples treated with 50 per cent or higher concentrations of carbon dioxide were quite dry and mealy, and they showed the first symptoms of "brown-heart." The apples in the control lots and those stored in 25 per cent carbon dioxide remained firm and juicy when held in air at 21° C. for one week, while the injured fruit had either dried up considerably or rotted.

#### *Banana*

Low temperatures and carbon dioxide treatments were very detrimental to green bananas during storage or subsequently when removed to warm air. As shown in Table III, the low temperatures without carbon dioxide tended to prevent good ripening of the fruit while the carbon dioxide treatments at these temperatures injured the fruit and favored rotting (Fig. 2). At temperatures higher than 10° C. the bananas ripened with good color and flavor and were apparently uninjured by 33 per cent carbon dioxide.

In storage at 15° and 21° C. the ripening processes were retarded to a slight extent by 15 to 25 per cent carbon dioxide and to a considerable extent by 33 per cent so that the bananas required two to three days longer than the controls to reach the soft ripe stage. Injurious effects of the treatment were not apparent with these concentrations as the fruit ripened with good color and flavor (Table III). Bananas held in 40 per cent carbon dioxide ripened very little during storage, but when removed to warm air the fruit ripened with a pale yellow color and an abnormal or flat taste. The effect of this treatment was similar to the observed effect of temperatures below 15° C. on untreated bananas. The abnormal or flat taste indicated the lack of starch conversion to sugar. The effect of the carbon dioxide treatment of bananas is similar to that reported by Geerligs (8) when the bananas stored in a nitrogen atmosphere without oxygen remained unchanged. The starch content of the pulp and the green color of the peel were retained. These abnormal conditions that were present to a slight degree in fruit treated with 40 per cent carbon dioxide were much more pro-

nounced in the fruit treated with higher concentrations of carbon dioxide at  $15^{\circ}$  and  $21^{\circ}$  C. (Table III). The green bananas held in this treatment lost the tannin flavor although the pulp remained starchy. Concentrations of 70 to 80 per cent carbon dioxide aided in rendering the fruit non-astringent, but prevented coloring, softening, or starch conversion (qualitatively determined by iodine method) while it was under treatment. Upon removal to warm air the pulp became very soft, and the peel turned brown before it would normally have lost its green color. The flavor of this fruit was decidedly insipid and somewhat alcoholic. If the fruit was over one-half ripe when placed in the highest concentration of carbon dioxide it became very

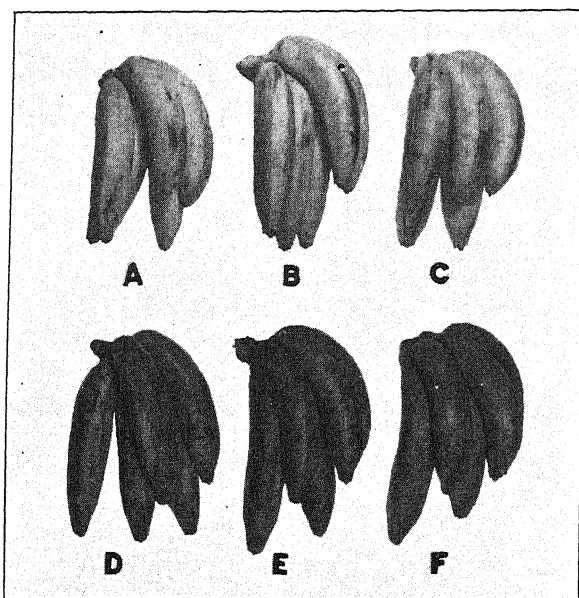


FIGURE 2. Bananas held in carbon dioxide for seven days at  $10^{\circ}$  C. A. Control in room, B. control in can, C. 25 per cent, D. 50 per cent, E. 75 per cent, and F. 83 per cent carbon dioxide.

soft and developed an alcoholic flavor while under treatment, and rotted readily when removed to air. The injurious effects were much more pronounced in fruit held at  $21^{\circ}$  C. than at  $15^{\circ}$  C. during the storage period.

#### *Peach*

As reported by Hill (11), the peaches that were held in a high concentration (30 per cent) of carbon dioxide for four days (Table II) acquired a decidedly bitter and nauseating flavor. Furthermore, the fruit did not soften during the carbon dioxide storage nor very rapidly after removal to

TABLE III  
EFFECT OF VARIOUS CONCENTRATIONS OF CARBON DIOXIDE ON GREEN BANANAS STORED AT DIFFERENT TEMPERATURES

Conditions for 7 day treatment		Observations at end of 7 day treatment	Observations made after the treated fruits had been held at 21° C. for the following additional periods	
Temp. ° C.	% CO <sub>2</sub> in can		4 days	9 days
4	Control in air " can 70	Green, firm " " "	Light green, softening, abnormal flavor " " " Brown, soft, alcoholic flavor	Rotten " "
	Control in air " can 25 50 70 80	Green, firm " " " " " " " " "	3/4 ripe, greenish yellow " 1/2 " Greenish yellow* Green, browning, soft Dark brown, very soft Black, very soft	Ripened to paler yellow than normal " " " Premature browning of peel, flavor as controls Rotting " "
15	Control in air " can 25 50 70 80	1/2 ripe " 1/2 " 1/3 Green " firm " "	Ripe, good color and flavor " " 4/5 ripe 1/3 colored Coloring, soft, abnormal flavor Coloring, very soft, abnormal flavor	Ripe, good color and flavor Ripe, color pale yellow, flavor abnormal Brown, very soft, alcoholic flavor " "
	Control in air " can 25 50 70 80	Ripe " 3/4 ripe Green, firm " " " "	Ripe, good color and flavor 1/2 colored, soft, abnormal flavor Coloring, soft, alcoholic flavor	Pale yellow, very soft, abnormal flavor Browning, very soft, alcoholic flavor " "

\* Few brown spots, \*\* Many brown spots.



warm air. Apparently the higher concentrations of the gas caused some chemical changes to take place in the skin of the peach since it became somewhat brown during storage. When removed to warm air the injured peaches browned very rapidly and soon rotted. The peaches that were held in the injurious concentrations of carbon dioxide were never so juicy as the untreated fruit except in the case of the fruit that had started to rot. Some concentrations of carbon dioxide (20 per cent at 0° and 4° C., and 10 per cent at 10° C.), as shown in Table II, did not cause noticeable injury to the peaches while in storage for four days. When removed to warm air at 25° C. these peaches apparently developed flavor comparable to the untreated fruit that was held under similar conditions of storage.

### *Pear*

The Anjou variety of pear was greatly injured by high concentrations of carbon dioxide while held in storage at 15° C. for seven days. As shown in Figure 3, the discoloration of the skin, as well as internal breakdown of

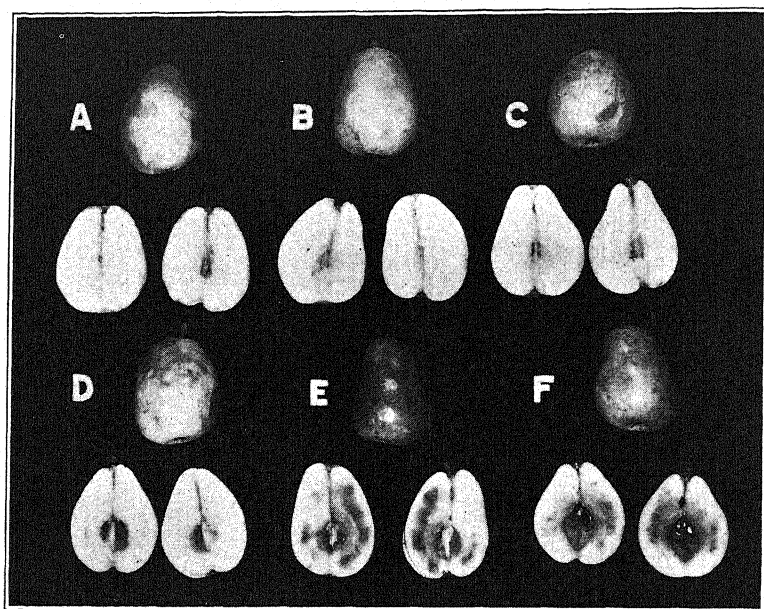


FIGURE 3. Anjou pears held in carbon dioxide for seven days at 15° C. A. Control in room, B. control in can, C. 25 per cent, D. 50 per cent, E. 70 per cent, and F. 80 per cent carbon dioxide.

the pear tissue exposed to 70 and 80 per cent carbon dioxide, was very noticeable. The pears exposed to 50 per cent carbon dioxide were browned somewhat at the center. The control pears remained firm with a slight

tannin flavor, while the pears exposed to 25 per cent or higher concentrations of carbon dioxide became soft, juicy, and lost the tannin flavor. These results with the pear are similar to those found by Gore (9), Lloyd (19), and others when processing persimmon fruit with carbon dioxide gas to render it non-astringent. However, under closely controlled conditions the processed persimmon fruit did not become soft as was observed with the pear. It is possible that if the conditions and period of treatments of the pears were rigidly controlled the fruit would lose its astringent flavor without becoming soft.

The data in Table II show that the Anjou pear (tested during January to March) was not noticeably injured. Softening occurred only with 42 and 25 per cent carbon dioxide at 0° and 4° C. respectively. Unlike the Anjou variety, the freshly harvested Bartlett pear was greatly injured by storage for seven days in 28 per cent carbon dioxide at all temperatures. The Bartlett pears in 10 per cent carbon dioxide were similar to the controls in color and firmness, but they were less astringent in flavor. During storage at 25° C. the Bartlett pears held in all concentrations of carbon dioxide became soft and started to rot. The control fruit held at this temperature (25° C.) developed some yellow color and began to soften, but it did not rot until one week after the treated fruit had been discarded.

#### *Strawberry*

Fulton (7, p. 15-17), in 1907, found that ripe strawberries held in triple-wrapped cartons produced 7 to 9 per cent carbon dioxide, softened, and developed a bad flavor during six days storage at 2.2° C. In contrast, experiments by Kidd and West, as given in the Great Britain Food Investigation Board, Fruit and Vegetable Committee (10, p. 18), have shown that ripe strawberries could be kept in good edible condition for three or four weeks if held at 1° to 2° C. in a container having an adjustable diffusion leak to retain the air containing a reduced amount of oxygen and a moderate amount of respired carbon dioxide. In tests being reported here the firm ripe strawberries were not noticeably injured by 15 per cent carbon dioxide when stored three days at 0°, 4°, or 10° C. However, the strawberries exposed to 25 per cent and higher concentrations of carbon dioxide (Table II) during storage, rapidly softened and developed an abnormal flavor and odor. The injured strawberries were inedible and rapidly rotted when held in warm air. The injurious effects of the carbon dioxide treatment were more noticeable on over-ripe and wet strawberries than on ripe firm, or three-quarters ripe dry or wet strawberries. The three-quarters ripe strawberries did not noticeably change in color or flavor after removal from the lower concentration (15 per cent) of carbon dioxide to warm air at 25° C. The three-quarters ripe as well as the ripe strawberries held in 15 per cent carbon dioxide did not show wastage any more rapidly than the

controls when removed from cold storage at 0° or 4° C. to warm air for two days.

### *Grapefruit*

Grapefruit treated with 25 per cent concentration of carbon dioxide (Table IV) developed no apparent injury either in storage or upon holding in air at 21° C. Grapefruit held in 50 per cent carbon dioxide developed a slight abnormal flavor that was noticeable only when closely compared

TABLE IV  
EFFECT OF VARIOUS CONCENTRATIONS OF CARBON DIOXIDE ON CITRUS  
FRUITS STORED AT DIFFERENT TEMPERATURES

Material	Days of storage	Per cent CO <sub>2</sub> at which no injury was observed at various temperatures in ° C.					Per cent CO <sub>2</sub> at which injury was observed at various temperatures in ° C.				
		0	4	10	15	21	0	4	10	15	21
Grapefruit											
Walters	7	25	25	25	25		50	50	50	50	
Foster											
Pink	7	50	50	50	50		65	65	65	65	
Thompson											
Seedless	7	50	50	50	50		65	65	65	65	
Orange*	7	50	50	50	50	50	64	64	64	64	64
Valencia	7	50	50	50	50		63	63	63	63	
King orange	7	†	30	30	†		27	52	52	30	
Tangerine	7	25	25	25	25		50	50	50	50	

\* Variety not known.

† Concentration of carbon dioxide not determined.

with the control fruit. With higher percentages of carbon dioxide there was developed a decided abnormal bitter flavor that was repugnant. This abnormal flavor developed by concentrations of carbon dioxide above 50 per cent was much more pronounced at 0° or 4° C. than at 10° or 15° C. Upon holding in warm air (21° C.) for 7 to 15 days the injured or uninjured carbon dioxide treated fruit did not change in flavor.

Subjecting the grapefruit to approximately pure carbon dioxide either by flowing the gas over the fruit or by reducing the air pressure, then releasing it with carbon dioxide, brought about the abnormal flavor very readily. Carbon dioxide flowing at the rate of one liter per hour into a four-liter container of grapefruit caused the development of an abnormal flavor within two days. Impregnating the intercellular spaces with carbon dioxide gas brought about the abnormal flavor within one day.

The pink flesh grapefruit, varieties Foster Pink and Thompson Seedless, showed no injury in 50 per cent carbon dioxide at the end of the storage period. Higher concentrations of carbon dioxide (Table IV) caused a slight abnormal flavor to develop during storage. After holding in air at 21° C. for 15 days the fruit was again examined. Fifty per cent carbon di-

oxide at 0° C. had resulted in no injury, but at higher temperatures there had developed an abnormal flavor and color of the grapefruit. Apparently this variety of grapefruit was slower to show the effects of the carbon dioxide injury.

#### *Sweet Orange*

The common or sweet oranges treated with 50 per cent carbon dioxide (Table IV) did not, in most tests, develop an abnormal flavor or show skin discolorations while in storage or after one week in air at 21° C. Occasionally individual oranges did develop a very slight abnormal flavor while in 50 per cent carbon dioxide. With high concentrations at all temperatures there was developed in the oranges during storage a decidedly abnormal flavor that was somewhat bitter and akin to over-ripeness. This injury developed to an equal extent in the carbon dioxide-treated oranges regardless of the storage temperature. There was an increase in the abnormal flavor of the injured fruit when it was held for one week in air at 21° C. Oranges held in 85 per cent carbon dioxide at 15° C. and 64 per cent at 21° C. were injured to the extent that they became very soft as a result of tissue breakdown. Higher concentrations of carbon dioxide (80 to 100 per cent) caused skin injury that did not fully develop until after 10 to 15 days in air at 21° C. The injured areas of the skin became dark in color, sunken, and hard. Special tests on the orange gave some results to indicate the rapidity of the carbon dioxide injury. Impregnating the intercellular spaces of the orange with carbon dioxide by reducing the air pressure and releasing it with carbon dioxide brought about a very decided abnormal flavor and odor within two days. Oranges subjected to flowing carbon dioxide developed a slight abnormal flavor within two days and a decidedly off-flavor by the end of the third day. Holding the oranges at 0° or 15° C. in approximately 100 per cent still carbon dioxide resulted in the development of a slightly abnormal flavor within three days. The abnormal flavor was greatly intensified by an additional day of the treatment. The skin injury due to high concentrations of carbon dioxide was quite pronounced and developed much more rapidly when increased oxygen was used. Increasing the oxygen content of the air in the cans before adding the carbon dioxide caused no apparent alteration in the injuries to the orange.

As shown in Table IV, the green skin Valencia orange was apparently not injured by 50 per cent carbon dioxide. Increasing the concentration to 65 per cent at any temperature did not alter the skin coloring and caused only a slight abnormal flavor to develop in the fruit. In no case was the flavor of the green skin Valencia oranges so abnormal as that of riper fruit similarly treated. The higher concentrations of carbon dioxide (70 to 80 per cent) injured the Valencia oranges, turning the skin somewhat brown and causing it to become very dry and hard, and to have an abnormal

flavor and odor. After normal coloring the flavor of the treated green skin oranges was not as sweet as that of the untreated green skin fruit that was allowed to color under the same conditions.

As reported by Barker (1), the oranges that were noticeably injured by high concentrations of carbon dioxide contained many white specks of various sizes on the outer membranes of the carpel walls (Fig. 4). These white specks were masses of hesperidin crystals that had precipitated out of the cell sap of the injured fruit. Tunmann (29, p. 371) pointed out that a loss of moisture from the cells of citrus fruit caused either by drying or placing in alcohol brought about the formation of the hesperidin crystals.

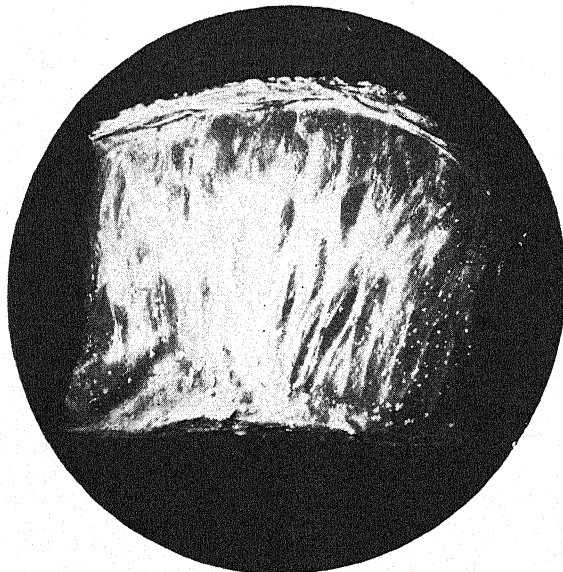


FIGURE 4. Hesperidin crystals on the outer membrane of the carpel wall of an orange that was held in 80 per cent carbon dioxide for seven days at 10° C. (Magnified four times).

Furthermore, Webber (30, p. 162) and Millikin and others (21, p. 257) invariably found hesperidin crystals present in citrus fruits that had been injured by freezing while on the tree. The hesperidin crystal formation in the orange tissue that was injured by either placing in alcohol or freezing takes place over a time interval of a few minutes to four or five days. In contrast, oranges treated with carbon dioxide exhibited crystal formation during the sixth or seventh day of treatment in storage or at any time up to 10 or 15 days after removal to air at 21° C. Oranges transferred from the carbon dioxide treatment to an atmosphere of approximately 100 per cent oxygen resulted in a more rapid and greatly concentrated formation of hesperidin crystals on the carpel walls. In Table V a comparison of results is

TABLE V  
 PRESENCE OF HESPERIDIN CRYSTALS ON THE CARPEL WALLS OF ORANGES THAT  
 WERE HELD IN VARIOUS CONCENTRATIONS OF CARBON DIOXIDE

Per cent CO <sub>2</sub> treatment	Hesperidin crystal formation after 7 days in carbon dioxide at the four temperatures given below and 7 days in air or oxygen at 21° C.							
	0° C.		4° C.		10° C.		15° C.	
	Air	O <sub>2</sub>	Air	O <sub>2</sub>	Air	O <sub>2</sub>	Air	O <sub>2</sub>
Control in air	o	o	o	o	o	o	o	o
“ “ can	o	o	o	o	o	o	o	o
28	+	o	o	o	o	++	o	++
50	+	o	o	++	o	++	o	++
68	++	++	++	++	++	++	++	++
79	++	++	++	++	++	++	++	++

o No crystals, + Few crystals, ++ Large quantity of crystals.

given for two methods of holding oranges after removal from carbon dioxide treatment. All concentrations of carbon dioxide used at 10° and 15° C. injured the oranges and the addition of oxygen brought about the crystalline formation at a more rapid rate.

#### *King Orange*

The range of concentration of carbon dioxide that was injurious to the King orange varied with the temperature of storage as shown in Table IV. At 0° and 15° C., 27 to 30 per cent carbon dioxide injured the fruit which resulted in the skin turning brown and a development of an abnormal flavor similar to that observed in tests with the common or sweet orange. The same concentrations of carbon dioxide at 4° and 10° C. caused no apparent change in the King orange. However, higher concentrations of carbon dioxide brought about a very decided injury to the King orange that resulted in abnormal flavor and skin discoloration with considerable softening of the skin and pulp tissues.

#### *Tangerine*

Tangerines were not injured by 25 per cent carbon dioxide during or after the seven day storage period at any temperature. Increasing the concentration of carbon dioxide to 50 per cent caused the development of an abnormal flavor, but no apparent injury to the skin. Tangerines held in higher concentrations of the gas were greatly injured, the skin and pulp tissue becoming soft, with abnormal flavor. When held in air at 21° C. this greatly injured fruit rotted very quickly while the slightly injured fruit became soft rather slowly.

#### EFFECT OF CARBON DIOXIDE ON VEGETABLES

##### *Asparagus*

Low concentrations of carbon dioxide (up to 28 per cent) retarded bud growth of the asparagus shoot. Higher concentrations of the gas (50 to 80

TABLE VI  
EFFECT OF VARIOUS CONCENTRATIONS OF CARBON DIOXIDE ON VEGETABLES  
STORED AT DIFFERENT TEMPERATURES

Material	Days of storage	Per cent CO <sub>2</sub> at which no injury was observed at various temperatures in °C.				Per cent CO <sub>2</sub> at which injury was observed at various temperatures in °C.			
		0	4	10	15	0	4	10	15
Asparagus	3	28	28	28	28	50	50	50	50
Bean, Stringless	3	*	18	18	*	18	30	30	18
Carrot (root)	5	80	80	80	25	*	*	*	50
Cauliflower	7	25	25	25	*	50	50	50	27
Celery	7	25	25	*	*	50	50	25	25
Lettuce	7	7	7	*	*	13	13	*	*
Mushroom	4	15	15	15	*	25	25	25	*
Pea	4	19	19	20	*	30	30	40	18
Radish (root)	7	50	50	25	25	70	70	50	50
Rhubarb (stalk)	7	80	80	80	80	*	*	*	*
" (dry leaf)	7	50	27	27	27	70	50	50	50
Spinach	4	25	*	*	*	30	20	20	20
Tomato	4	6	6	6	*	10	10	10	6

\* Concentration of carbon dioxide not determined.

per cent), as shown in Table VI, caused injury resulting in either the browning of the outer bud scales or the development of water-soaked areas of a dark green color. Treated buds showing injury became soft, whereas the untreated ones remained firm. Further injury brought about by high concentrations of carbon dioxide resulted in the development of a flabby tough shoot that failed to break with a snap as did the controls. Increasing the concentration of carbon dioxide and raising the temperature, produced a correspondingly greater injury. Furthermore, increasing injury occurred accordingly when the freshly cut ends of the stalks were held in air, in contact with moist sphagnum moss, and in water.

#### *Bean and Pea*

The response of stringless beans and green peas to storage in carbon dioxide was variable as shown in Table VI. These results indicate that the effect of a given concentration of the gas depends upon the age of the vegetable and the storage temperature. Moist hulls were more susceptible to carbon dioxide injury than dry hulls. This condition may be explained by the suggestion of Kidd, West, and Kidd (18, p. 23) to the effect that water on fruit in carbon dioxide storage might be harmful in that it provided better conditions for fungous invasion and may influence gaseous exchanges between internal and external atmospheres of the fruit. The injury to beans and peas brought about by the carbon dioxide treatment resulted in the appearance of dark bruised areas on the hulls and the production of an abnormal flavor in the peas. The degree of the injury increased with in-

creasing concentrations of carbon dioxide and with a rise in temperature, being least at 0° and 4° C. When removed from the treatment to air at 25° C., fungous growth on the injured tissue took place very rapidly while the control and the treated beans and peas that were uninjured remained in good condition.

#### *Carrot and Radish*

Data in Table VI show that the carrot root withstood a much higher concentration of carbon dioxide than the radish during storage at 0° or 4° C. Carrot roots were injured, however, when held in storage at 15° C. with 50 per cent carbon dioxide, the crown becoming soft and the root soon rotting. When injured by the gas treatment there appeared soft watery blotches on and under the skin of the radish root as well as the development of an abnormal odor and flavor. Usually fungous growth appeared on the injured radish tissue after removal to air at 25° C. Carrot and radish leaves were greatly injured by all concentrations of carbon dioxide used. After removal from storage the uninjured roots with injured leaf tissue did not remain in good condition as long as the roots from which the injured leaves were removed.

#### *Cauliflower*

A concentration of 25 per cent carbon dioxide at 0° and 10° C. brought about no apparent change in the cauliflower. However, increasing the temperature to 15° C. caused the development of some injury by 27 per cent carbon dioxide. Higher concentrations at all temperatures caused injury that resulted in softening of the buds and abnormal odor. After removal to air at 25° C. the injured tissue rotted in one or two days while the control and uninjured carbon dioxide treated cauliflowers remained in good condition.

#### *Celery*

Carbon dioxide injury to celery stalks and leaves resulted in a softening of the tissue that was brown in color and very watery. In a short time this injured tissue rotted with an offensive odor. The amount of injury varied with the concentration of carbon dioxide and the temperature at which the celery was held during storage. As shown in Table VI and Figure 5, 25 per cent carbon dioxide did not injure the celery stalks when held seven days at 0° or 4° C. When removed to warm air (21° C.) for four days, the celery held at 0° C. showed slight browning of the stalks while that held at 4° C. developed 5 per cent loss due to rot. As shown in Table VII, the loss of the celery due to rot as a result of carbon dioxide injury was considerable when held at a high temperature for four days. Celery stored at 0° C. in 50 to 80 per cent carbon dioxide was injured to a greater extent than celery stored in the same concentrations at 4° C., but upon removal to



warm air the celery from both storage temperatures broke down to the same extent.

Celery held at 15°, 21°, and 31° C. was greatly injured by all concentrations of carbon dioxide used. With the higher concentrations (70 to 80 per cent) of carbon dioxide at these temperatures the celery was reduced to a rotten watery mass. The room controls at these temperatures wilted greatly, but were easily freshened by placing in water. Control lots in cans started to rot, due possibly to excessive moisture at the high temperatures, but in no case to the same extent as the treated celery.

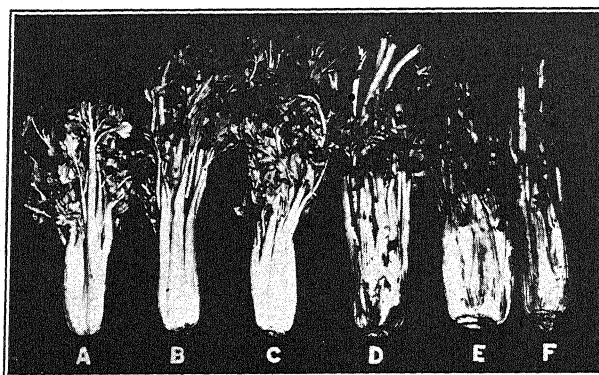


FIGURE 5. Celery held in carbon dioxide for seven days at 0° C. A. Control in air, B. control in can, C. 25 per cent, D. 50 per cent, E. 70 per cent, and F. 80 per cent carbon dioxide.

TABLE VII  
ESTIMATED PERCENTAGE OF INJURY AND ROT OF CELERY AFTER STORAGE  
IN CARBON DIOXIDE AT VARIOUS TEMPERATURES

Temperature ° C.	Concentration of CO <sub>2</sub> per cent	Condition at end of 7 days stor- age in CO <sub>2</sub>	Condition after an additional 4 days in air at 21° C.
0	Control	Good	Good
	25	"	Slight discoloration of stalk
	50	Slight injury	40% loss due to rot
	70	10% "	50% " " " "
	80	15% "	75% " " " "
4	Control	Good	Good
	25	"	5% loss due to rot
	50	1% injury	25% " " " "
	70	1% "	50% " " " "
	80	5% "	75% " " " "
10	Control	Good	5% loss due to rot
	25	Some browning	10% " " " "
	50	1% injury	40% " " " "
	70	5% "	50% " " " "
	80	25% "	Total " " " "

An attempt was made to retard the development of the carbon dioxide injury to the celery by increasing the oxygen content of the air in the storage cans to 36 per cent before the addition of the carbon dioxide. The results as given in Table VIII show that during storage 85 per cent carbon dioxide caused only slight injury to the celery which resulted in 10 per cent

TABLE VIII  
INFLUENCE OF CARBON DIOXIDE ON CELERY AFTER RAISING THE INITIAL  
CONCENTRATION OF OXYGEN TO 36 PER CENT

Final concentrations		Condition at end of 7 days storage at 4° C.	Condition after an addi- tional 2 days in air at 21° C.
Per cent CO <sub>2</sub>	Per cent O <sub>2</sub>		
0	36	Good	Good
25	19	" "	"
50	11	"	"
67	12	Slight injury to leaves	Slight softening of stalks
85	6	" " " " " and stalks	10% injury and loss due to rot

loss when it was held for an additional period in warm air. In contrast, the results in Table VII show that 80 per cent carbon dioxide without additional oxygen caused 5 per cent loss during storage and 75 per cent loss when it was held for an additional period in air. From these results it appears that some of the injury was due to insufficient oxygen in the storage atmosphere.

#### *Lettuce*

Lettuce kept moist during storage was readily injured by concentrations of carbon dioxide above 7 per cent as shown in Table VI. The injury developed was identical with that observed in the case of the celery where the tissues broke down and soon rotted. Hard-head lettuce became soft, lost crispness, and soon rotted when exposed to an injurious concentration of carbon dioxide. The injury was the same type in all tests but was much more pronounced with increased temperature and concentration of carbon dioxide.

#### *Mushroom*

Data in Table VI show that mushrooms withstand 15 per cent, but are slightly injured by 25 per cent carbon dioxide when held in storage for four days. Injuries brought about by the gas consisted of browning of the pileus and stipe in addition to some softening of the stipe. Mushrooms stored in all concentrations of carbon dioxide, but more especially above 15 per cent, developed abnormal flavor and odor that was similar to the odor of green corn husks. This abnormal flavor and odor completely left the carbon dioxide-treated mushrooms when held in warm air for two to three hours. Even when greatly injured the mushrooms did not rot but instead dried up when held from three to four days after the treatment.

*Rhubarb*

Rhubarb stalks were not easily injured by the carbon dioxide gas. The petioles withstood as high as 80 per cent carbon dioxide for seven days without showing injury even upon holding at 25° C. for two to three days after the treatment. The leaf tissue when held dry was injured by a concentration of 50 per cent carbon dioxide. The injurious effect of the treatment was greatly augmented by the presence of moisture on the surface of the leaf. The injury to the leaf tissue resembled softening and watery breakdown as observed with celery or lettuce.

*Spinach*

Spinach was injured by relatively low concentrations of carbon dioxide (Table VI). Although 25 per cent carbon dioxide did not injure the spinach while in storage at 0° C., all other concentrations at this and higher temperatures caused considerable injury to the leaf tissue. In all cases the injury developed was a softening of the tissues accompanied by an abnormal odor. When removed to air the injured tissue soon rotted. Dry leaves were less injured by the carbon dioxide treatment than those with moisture on the surface.

*Tomato*

The tomato (Table VI) was very susceptible to the injurious effects of the carbon dioxide gas when held in storage for any length of time, being injured by 10 per cent carbon dioxide at 0° and 10° C. The gas tended to bring about a complete softening of the carpel walls resulting in a watery mass of abnormally flavored pulp enclosed in a somewhat tough skin. The riper the tomato the greater was the carbon dioxide injury.

*Potato*

Irish Cobbler potatoes of the 1929 crop harvested about July 15 were held at room temperature, 24° to 27° C., until July 30. The stock of potatoes was divided into two portions; one was placed under treatment immediately and the other portion held at room temperature until September 13, then placed in carbon dioxide storage. The potatoes were held in the tin cans with various concentrations (10, 20, 50, and 80 per cent) of carbon dioxide at temperatures of 4°, 10°, and 25° C. for a period of 34 days during each test. Two control lots of potatoes were used in each test. In the first test none of the tubers sprouted because they were dormant, whereas during the second test the control tubers held at 25° C. sprouted readily. The two lower temperatures (4° and 10° C.) retarded the sprouting of the tubers in the second test.

*Dormant tubers.* Potatoes in carbon dioxide storage at 4° C. were not apparently injured by the treatment. However, cut pieces showed an ab-

normal streaked appearance in the pith. This streakiness appeared as water-soaked, star-shaped areas which were considerably larger than those normally found in potato tubers. At 10° C., 10 and 20 per cent carbon dioxide-treated tubers displayed the abnormally streaked areas in the pith cells. Potato tubers held in high concentrations of carbon dioxide showed considerable blackheart. These tubers were blackened even to the skin so that in most cases the injured tubers could be removed from the good without their being cut. These results are in agreement with those of Stewart and Mix (25), that tubers affected with blackheart produced by exclusion of air showed surface discoloration.

Potato tubers treated with 10 per cent carbon dioxide at 25° C. were not different from the controls. Those treated with 20 per cent showed the abnormal streakiness of the pith cells. Tubers held in higher concentrations of carbon dioxide were injured considerably. Besides the abnormally streaked areas in the pith cells there was some softening of the cortex layer that appeared brown in color. The injured potato tubers rotted very quickly upon removal to air.

Potato tubers removed from carbon dioxide storage and held for 51 days in air at 25° C. started to sprout in all cases except where decay or blackheart occurred. As reported by Kidd (12), the size of the sprouts varied inversely with the concentration of carbon dioxide used and was in all cases smaller than those of the control tubers.

*Non-dormant tubers.* Potato tubers held in air for 60 days, then placed in various concentrations of carbon dioxide at 4°, 10°, and 25° C. for 34 days, showed considerable injury in most cases. Ten per cent carbon dioxide at 4° C. caused no apparent change in the tubers, but those held in this concentration at 10° C. developed a pink color in the pith cells upon exposure to air. Potato tubers that were treated with 20 to 64 per cent carbon dioxide at 4° or 10° C. showed definite blackheart. As shown in Figure 6, the injury to potato tubers resulting in blackheart increases with increasing concentrations of carbon dioxide.

Increasing the temperature of the storage room brought about a change in the observed carbon dioxide injury to the potato tubers. Treatments with 14 to 30 per cent carbon dioxide resulted in the tubers having somewhat of a browned external appearance and in most cases developing a brown rot at the stem end. Increasing the concentration of carbon dioxide to 50 to 65 per cent brought about considerable surface browning, brown rot, and blackheart of all potatoes held in the treatment.

Sprouting of the tubers was not entirely prevented by 14 to 30 per cent carbon dioxide. The size and condition of the sprouts on tubers held in 19 and 30 per cent carbon dioxide were decidedly inferior to either of those of the control or 14 per cent treated tubers.

*Respiration of potatoes in various concentrations of carbon dioxide.* Ninety-

one kilograms of Irish Cobbler potatoes harvested about July 15 were placed in each of three 208-liter steel drums. This quantity of tubers filled the drum within 15 cm. of the top. The gas sampling tubes and cables of the thermometers were inserted to various depths in the drums. The cover and other openings were sealed with plasteline clay. The steel drums were placed in a box and covered all over with 15 to 20 cm. of finely ground cork to provide heat insulation. The whole apparatus was set up in a warm room where the temperature was controlled as nearly as possible to 21° C. by cold air ventilation.

The potato tubers were held in these steel drums under three concentrations of carbon dioxide: without added carbon dioxide, with 12 per cent,

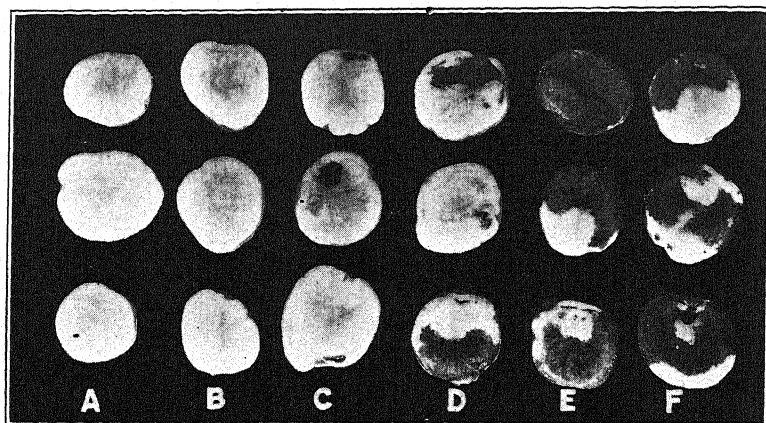


FIGURE 6. Potatoes stored in carbon dioxide for 34 days at 10° C. A. Control in can, B. 10 per cent, C. 20 per cent, D. 32 per cent, E. 47 per cent, and F. 64 per cent carbon dioxide.

and with 79 per cent carbon dioxide added the first day of storage. Daily analyses of the carbon dioxide produced by the potatoes in each of the three drums were made and the data in form of curves are shown in Figure 7. Whether or not carbon dioxide was added, the respiration of the potato tubers continued at about the same rate at the lower concentrations. Higher concentrations of carbon dioxide tended to slow down the rate of carbon dioxide production.

Analyses of the oxygen content of the air about the tubers were made daily. The oxygen analyses for the lower carbon dioxide content drums are shown in Figure 7. There was no oxygen present in the drum that had 79 per cent carbon dioxide added. The data represented by the curves, drawn to the same scale, show a much more rapid consumption of oxygen than production of carbon dioxide by the potato tubers.

Changes in the temperature of the potatoes during storage amounted only to a rise of one to two degrees Centigrade.

The potato tubers held in the steel drums both with and without added carbon dioxide were in very poor condition. Tubers held in the steel drums

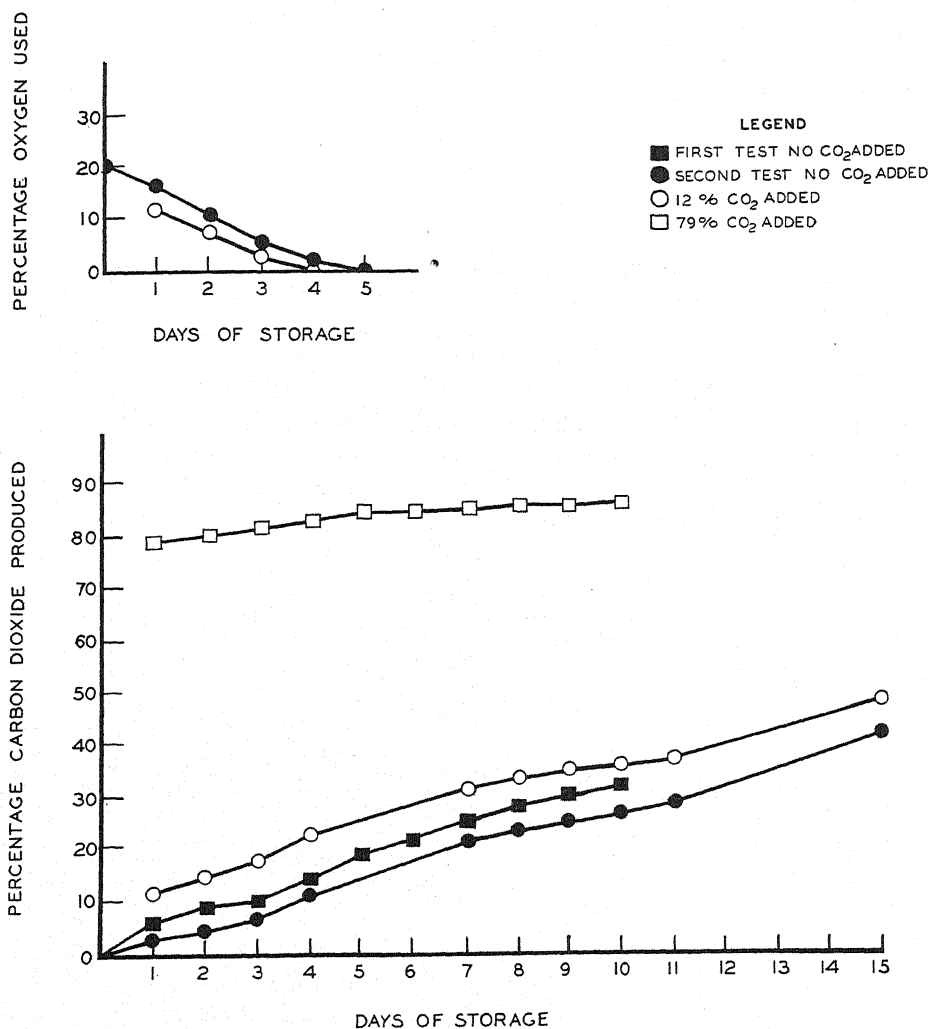


FIGURE 7. Respiration of potatoes in closed storage at 21° C.

without added carbon dioxide for 10 days showed considerable rot, while those held for 15 days showed both rot and blackheart. The potato tubers held with added carbon dioxide showed both blackheart and a brown rot at the end of 10 days of storage.

## DISCUSSION

The results of tests for all fruits and vegetables, except the grapefruit, show that increasing the temperature at any given concentration of carbon dioxide will increase the injury. The grapefruit was found to be greatly injured by a high concentration of carbon dioxide when held at  $0^{\circ}$  or  $4^{\circ}$  C., but only slightly injured by the same concentration when held at  $10^{\circ}$  or  $15^{\circ}$  C. These results should be further investigated to determine the cause of such a variation in the reaction of these fruits to the effect of carbon dioxide storage.

Determinations were made on the percentage of oxygen present during the storage of fruits and vegetables in various concentrations of carbon dioxide. Because of insufficient time available the effect of oxygen on the stored material was not studied in detail. From the results of many investigators as heretofore discussed, it is evident that the oxygen content of the atmosphere about the stored material must not be too low or injury will result. As already shown, the addition of oxygen to the atmosphere about celery stored in a high percentage of carbon dioxide greatly decreased the injury during storage. However, additional oxygen content about citrus fruit during storage apparently had no effect on the injury. From these widely different results it is apparent that additional research is necessary to establish definitely the relation between the concentrations of oxygen and carbon dioxide that bring about injury to fruits and vegetables while in storage.

## SUMMARY

Many workers have reported incidental observations and specific researches dealing with the effect of carbon dioxide on some fruits and vegetables that were held in one or two temperatures of storage. For the most part these experiments have been duplicated. In this paper there is reported the effect of carbon dioxide on many varieties of fruits and vegetables during storage for three to seven days at temperatures of  $0^{\circ}$ ,  $4^{\circ}$ ,  $10^{\circ}$ ,  $15^{\circ}$ ,  $21^{\circ}$ , and  $25^{\circ}$  C. Additional results are given for the condition of the fruits and vegetables after removal to air at  $21^{\circ}$  to  $25^{\circ}$  C. for a period from 1 to 14 days. The results of these experiments may be summarized as follows:

1. Effect of low concentrations of carbon dioxide during storage: (a) retarded respiration; external color changes and in some cases noticeable chemical changes associated with ripening (banana and peach); (b) removed the noticeable astringency found in some green fruits (banana and pear).
2. Effects of high concentrations of carbon dioxide during storage: (a) impaired the flavor of all fruits and some vegetables; (b) inhibited ripening; prevented changes in color and flavor (banana, peach, and orange);

(c) killed growing tissues (asparagus and potato sprouts), and promoted decomposition of exposed tissues that were high in moisture content (most vegetables); (d) internal discoloration and breakdown (apple, pear, peach, potato, and tomato); (e) external discoloration (pear, peach, and banana).

3. Observations on fruits held at 0°, 4°, and 10° C. showed that the strawberry in three days storage was greatly injured by 25 per cent concentration, whereas the Delicious apple in seven days storage was only slightly injured by 70 per cent concentration of carbon dioxide.

4. Experiments with citrus fruits showed the range of injurious concentrations of carbon dioxide during seven days storage at 0° to 15° C. to be 50 per cent for tangerines and grapefruit, and 64 per cent for oranges.

5. The flavor of the Walters variety of grapefruit was greatly impaired by 64 per cent carbon dioxide during storage at 0° or 4° C., but the flavor was only slightly impaired by the same concentration at 10° or 15° C. This result was contrary to those of all other materials tested where the injury increased with increasing storage temperature.

6. The range of injurious concentrations of carbon dioxide on vegetables during storage at 0°, 4°, and 10° C. varied from 10 per cent on tomatoes in four days storage to 50 per cent on asparagus in three days storage, and cauliflower, radish, and rhubarb leaves in seven days storage. Carrot roots and rhubarb petioles withstood as much as 80 per cent carbon dioxide for five and seven days respectively in storage.

7. In general the percentage of carbon dioxide necessary to cause injury to fruits and vegetables during storage is related directly to the firmness of the plant tissue and inversely to the amount of moisture on its surface.

8. The results indicate that for rapid respiring material the oxygen content of the atmosphere may be the controlling factor in so-called carbon dioxide injury. Further studies are necessary to determine definitely the relationship between the oxygen and carbon dioxide content of storage atmospheres in which either fruits or vegetables become injured during short periods of storage.

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# SOME EFFECTS OF ULTRAVIOLET RAYS ON THE VITAMIN D CONTENT OF PLANTS AS COMPARED WITH THE DIRECT IRRADIATION OF THE ANIMAL

MARY LOJGIN<sup>1</sup>

This is a study of the curative effects of ultraviolet radiation on the diseased condition of the animal known as rickets, which is brought about by a deficiency of vitamin D. The purpose of the study was to determine first whether there is an appreciable difference in the antirachitic properties of green plants exposed either continuously or for a short period of time to the extreme ultraviolet region of sunlight, or artificial source, which is not transmitted by ordinary greenhouse glass. A secondary consideration was the minimum daily exposure necessary to protect rats when irradiated directly as compared with the minimum exposure of green plant tissue, which when subsequently fed would protect rats in a similar manner. A further study was made of the antirachitic potency of plants irradiated while living and still attached to the roots as compared with plants cut off and irradiated after a definite time period.

## DISCUSSION OF LITERATURE

Hess and Weinstock (10), Hess, Weinstock, and Helman (12, 13), Steenbock (27), and Steenbock and Nelson (29) found that various foods, especially oils, which in a natural state possess no antirachitic properties, can be rendered active in this respect by means of ultraviolet radiation. Bills, Honeywell, and MacNair (2) and Rosenheim and Webster (22) have shown further that the antirachitic factor in these substances is confined to the nonsaponifiable fraction, and that it is an impurity closely associated chemically with cholesterol which these substances contain. Work by Rosenheim and Webster (23) and Windaus and Hess (30) has shown this impurity to be much more potent antirachitically than the preparation of cholesterol from which it had been separated. The active compound was identified as ergosterol, originally isolated from ergot, which upon irradiation with ultraviolet has great potency in the cure of rickets. Cholesterol was found to contain about one part in 2,000 of the pro-vitamin ergosterol. Activated ergosterol protects rats against rickets in daily doses of 0.0001 milligrams, and five milligrams were found to be approximately equal to one liter of good cod liver oil in antirachitic value.

In view of this fact it appears that any substance which contains ergosterol can be activated to some extent by irradiation with ultraviolet, and

<sup>1</sup> The author is indebted to the Corning Glass Works, Corning, New York who furnished both material and funds for this study and to Dr. John M. Arthur for his valuable suggestions and criticism of the work.

that there is a difference only in the degree of antirachitic potency which is developed. The question arises as to whether green vegetables irradiated with ultraviolet contain a sufficient quantity of activated ergosterol to render them antirachitically potent.

In case of direct irradiation of the animal the curative effect of ultraviolet radiation is without doubt due to the activation of ergosterol contained in the skin, since this radiation does not penetrate the tissue deeply. The fact that ergosterol is present in the epidermis in a concentration sufficient to render irradiated skin a rich source of vitamin D has been proved by Hess and Weinstock (11). They found that a dose of one gram per day of irradiated human or calf skin fed to rats on a low phosphorus diet would regularly protect them from rickets. It can be assumed, therefore, that the degree of protection imparted to an animal by ultraviolet radiation depends upon the amount of exposure of the epidermal layers.

A number of experiments have been carried on for the purpose of determining the antirachitic value of different plants. Hess and Unger (8) found that 30 grams of spinach daily did not protect infants from rickets. Hess and Weinstock (9) report that Zucker failed to prevent rickets in young rats when feeding them spinach *ad libitum*. Shipley, Kinney, and McCollum (26) found no antirachitic value in cabbage and spinach and a definite antirachitic value in dried alfalfa when administered to rats in the form of an ether extract. Zucker and Barnett (31) reported an absence of antirachitic value in extracts of carrots and spinach leaves. Goldblatt and Zilva (6) could not cure rickets with a dose of three grams of fresh spinach per day; neither could McClendon and Shuck (18) when feeding spinach in quantities varying from 0.01 per cent to 75 per cent of the diet.

On the other hand, Hess and Weinstock (11) succeeded in rendering spinach antirachitically potent by irradiating it with the mercury vapor lamp. These investigators reported also (9) that wheat grown in the dark had no antirachitic potency, whereas wheat grown in the light and irradiated with the mercury vapor lamp for one hour per day gave protection when fed to rats in a dose of ten grams per day. The same was true of lettuce; green lettuce leaves from the market were of no value in preventing rickets, whereas after irradiation for one hour ten grams per day would supply a rat with a sufficient amount of vitamin D.

Steenbock and Black (28) found that alfalfa and clover hay vary in antirachitic properties according to the amount of previous exposure to light. It was also reported (7) that the addition of fresh green oats increased the calcium retention of a goat, while raw cabbage did not seem to have any such effect.

Chick and Roscoe (3) found a variation in the vitamin D content of spinach grown during different seasons of the year. They reported that spinach grown in the open in winter, spring, and autumn possessed no anti-

rachitic properties, whereas spinach grown in midsummer had a slight but appreciable antirachitic value. They also called attention to the fact that summer spinach grown in the open is more effective in increasing the calcification of the bones than spinach grown under glass. They found that spinach leaves irradiated with the mercury vapor lamp became powerfully antirachitic.

#### EXPERIMENTAL METHODS

The animals used in this experiment were, in general, white rats, *Rattus norvegicus*, reared at this Institute, four to five weeks old and weighing from 40 to 70 grams. The only exception to this was during the first experiments when a large number of experimental rats were needed, and the breeding colony was not producing at a sufficient rate. Young rats purchased from commercial sources were fed the stock ration used for the breeding colony for at least two weeks before starting them on experimental diets. Other work (14) has shown the importance of having the diet controlled not only throughout the experiment, but also during the pre-experimental period. By the end of a period of two weeks these rats did not differ in weight and size from those reared at this Institute; and microscopic examination and ash percentage of bones showed no consistent differences.

The breeding colony was fed Sherman's (25) stock ration B (diet 13), and in addition to this some meat and lettuce was given daily. Young rats, after they had reached the age of 21 days, were not permitted to eat the meat fed regularly to the breeding colony, since consumption of a diet rich in phosphorus during the pre-experimental period might influence the results in subsequent experiments.

The experimental rats were kept in individual metal cages with raised bottoms in a totally dark room, and were fed ad libitum Sherman and Pappenheimer's diet 84 (24) and distilled water. They were weighed every week. A record was kept of the amount of food consumed. This is important as it has been found that fasting has a healing effect upon rachitic bones (19). All rats which consumed less than 28 grams of food per week were rejected in order to be sure that no curative effect in the experiments had been caused by starvation.

Each experiment lasted 35 days. At the end of this time rats on Sherman's diet 84 developed very severe rickets, unless supplied with vitamin D from some other source. During the pre-experimental period all the rats had stored enough vitamin A to last them until the end of the experiment; consequently no trouble with ophthalmia was observed, although the rickets-producing diet used is very deficient in vitamin A.

After the experiments had been under way for some time, a considerable difference was noticed between the growth curves of those rats which received the basal diet only, and those which were fed some plants in addi-

tion. While the latter in most cases gained steadily, the former did not, and even had a tendency to lose in weight. This may be expected since young rats grow very little on such a restricted diet. The plant tissue supplied additional factors, which produced normal growth. This fact, however, complicated the nature of the studies. Rickets has been defined as a state in which, as a result of lowered ion concentration,  $\text{Ca}_3(\text{PO}_4)_2$  is deposited too slowly to keep pace with new bone production and growth and consequently poor calcification results (15). It is apparent that if a rat gains considerably in weight, its bones grow rapidly and consequently a large rachitic metaphysis consisting of osteoid tissue and uncalcified bone should be formed. On the other hand, if a rat does not grow, no new uncalcified bone is formed, and hence no rickets should be produced. The latter is not exactly the case, for all control rats on diet 84 in these experiments grew very little but developed severe rickets. It is considered probable, however, that the more an animal grows, the greater the amount of antirachitic factor needed to form normal bones. In case the growth curves of the experimental animals varied greatly it was found impossible to judge, by comparing the calcification of their bones, the amount of antirachitic factor received by them.

To overcome this difficulty it was thought advisable to change the basal ration in such a way as to get more uniform growth curves. Since it was observed that lettuce grown under window glass possesses no antirachitic value, it was decided to feed greenhouse lettuce daily to all the experimental rats which were getting basal diet only. This improved considerably the growth curves of the rats without decreasing the rickets-producing properties of the diet.

At the end of the experimental period the rats were killed by means of chloroform. The distal end of a femur and the proximal end of a tibia were examined histologically, using McCollum's silver nitrate line test (20). This consists of the following: the bones are split in two, kept in acetone for 48 hours, then immersed in a 1 per cent silver nitrate solution, after which they are exposed to sunlight or to the light of an arc lamp and studied through a microscope. The deposits of calcium salts are stained brown by silver nitrate, and the metaphysis if present appears as a white band, the width of which depends on the degree of rickets developed.

The other femur was used for the ash percentage determination employing the method of Bethke, Steenbock, and Nelson (1). The dry femur was scraped free of adhering tissue, crushed, and extracted with alcohol and ether. This extraction is considered necessary as the bones of animals vary a great deal in lipid and fat contents, even when taken from animals on the same diet. The extracted bones were dried, weighed and then incinerated in an electric muffle furnace for four hours. Percentage of ash was calculated on the basis of the dry extracted bone.

In order to make the experiments as nearly quantitative as possible an attempt was made to compare the degree of rickets developed by each rat by measuring the width of the rachitic metaphysis. This was carried out in the following way: a camera lucida drawing of a longitudinal section of a femur and of a tibia of each rat was made, particular care being taken to keep constant the ratio of the actual size of the bone to its size on the drawing. By measuring the area of the metaphysis with a planimeter and dividing the results by the length of the area, the average width of the metaphysis was calculated from the drawing. The figures obtained in this way have no absolute value, but can be used to compare the degree of rickets developed and, therefore, the degree of protection afforded each rat in the various treatments.

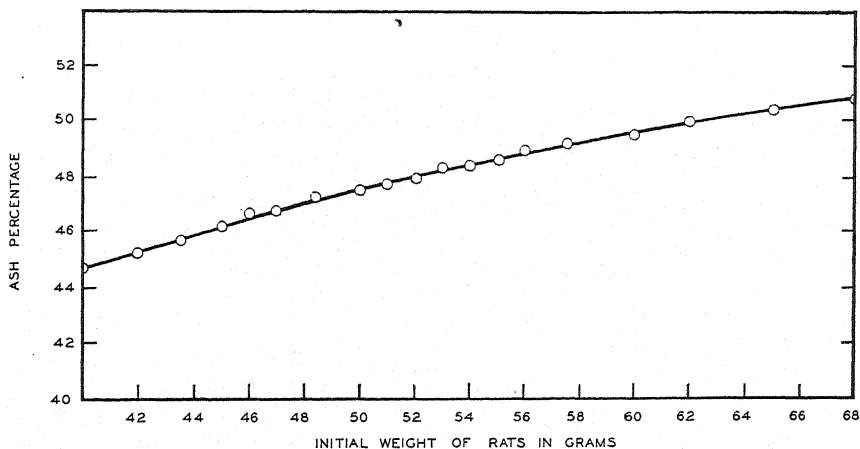


FIGURE 1. Relation of average ash percentage of femurs to initial weights of normal rats.

The other criteria used for the quantitative determination of the degree of rickets developed by an experimental rat was the deviation of the ash percentage of the femur from that of a normal rat of the same weight. It has been reported by several investigators that ash percentage of the bones varies with the size and age of the rat (5). A curve was plotted showing the relation of the ash percentage of the femurs to the weight of rat at the beginning of the experiment of over 200 rats receiving Sherman's diet 84 plus an adequate amount of antirachitic factor (Fig. 1). The deviation figures were obtained by comparing the ash percentage of a femur of an experimental rat with the average ash percentage determined from the curve for protected rats of the same size.

#### EXPERIMENTS WITH PLANT TISSUE

The plants used for these experiments were lettuce (*Lactuca sativa* L.), alfalfa (*Medicago sativa* L.), spinach (*Spinacia oleracea* L.), New Zealand

spinach (*Tetragonia expansa* Murr.), cabbage (*Brassica oleracea* var. *capitata* L.), swiss chard (*Beta vulgaris* var. *cicla* L.), and soy bean (*Glycine* Max Merr. Peking variety). Four grams of fresh plant tissue were fed to each rat daily. This dose was adopted as the maximum amount of tissue consumed more or less easily by all animals. Some rats would easily have eaten a much larger portion of certain plant tissue; but in other cases it was difficult to make some individuals eat even four grams. Feeding of a uniform dose in all cases made it possible to compare the results obtained with different plants.

Two different sources of ultraviolet rays were used, (a) sunlight and (b) the light of a Cooper Hewitt mercury vapor arc lamp in quartz. In the first case, plants were grown in the open and under filter N (a special ultraviolet transmitting glass) and were compared as to vitamin D content with plants grown similarly under ordinary greenhouse glass. The three growth conditions were as follows:

1. Under ordinary greenhouse glass which cuts out all rays shorter than wave length  $312\text{ m}\mu$ .
2. Under filter N which transmits when new about 80 per cent of the incident radiation at wave length  $290\text{ m}\mu$ .
3. In the open sunlight.

The transmission of new filter N and of filter S exposed to quartz mercury arc, and of greenhouse glass is shown in Figure 2.

As seen from Tables I, II, and III, plants grown in open sunlight had little antirachitic value, as rats developed rickets even when fed ad libitum plants grown in sunlight. A slight improvement was noted, however, in the calcification of the bones of the rats receiving soy beans, New Zealand spinach, lettuce, and alfalfa grown outside and under filter N, as compared with those receiving the same plants grown in the greenhouses. Cabbage and swiss chard, on the other hand, had no antirachitic value when grown either in the open or under filter N. None of the plants listed above were found to have any antirachitic value when grown under greenhouse glass.

In the case where a mercury vapor arc lamp was used as a source of ultraviolet rays, an attempt was made to determine the following points:

1. What dose of radiant energy is necessary to render cut or intact plants antirachitically potent, using different regions of the ultraviolet rays as transmitted through various filters.
2. Whether the antirachitic substance formed in the living plant by irradiation is destroyed after a certain period of time.
3. Whether several short periods of irradiation of the living plant are more effective than a single long period.

The transmission of various filters used in this work is shown in Figure 2 and the wave lengths at which each filter transmitted 1, 10, and 50 per cent are indicated in Table IV.

As seen from Tables I, II, and III, irradiation with the mercury vapor lamp formed an appreciable amount of antirachitic vitamin in all plants used in this work, with the exception of cabbage. Thirty minutes exposure of the cut plant to the rays from the lamp transmitted through filter N had no effect on cabbage, very slight effect on spinach, and a very noticeable effect on swiss chard. The same exposure to the rays transmitted through filter A rendered lettuce and alfalfa adequately antirachitic. The positive results obtained with lettuce and alfalfa, as compared with the other plants used, may be partially due to the higher response to ultraviolet radiation in these two plants; but they are, also due to the fact that a better filter was used in this experiment. Filter N, which was used in the earlier experiments, becomes solarized from exposure to the mercury vapor lamp and loses much of its transmission after a short exposure.<sup>2</sup> The percentage transmission of this filter when new and after solarization has been determined by Coblenz and Stair (4). New filter N has a transmission of 10 per cent at approximately wave length  $218\text{ m}\mu$ . After 75 hours exposure to the mercury arc the same glass has a transmission of 10 per cent at approximately wave length  $302\text{ m}\mu$ . The solarized glass was replaced by new glass as soon as the slightest signs of solarization were noticed, but the actual transmission of this filter was lower on the average than that of the new filter N. As pointed out by Coblenz and Stair the amount of solarization could be determined roughly by the degree of pink color developed in this filter. Filter A, on the other hand, is not affected appreciably by the rays of the mercury vapor lamp. The better quality of filter A is well illustrated by the fact that there was a much larger amount of antirachitic factor formed in lettuce irradiated through filter A for 13 minutes than in lettuce irradiated through filter N for 20 minutes (Table II).

It can be seen from Tables II and III that cut plants have a higher response to irradiation than intact plants. Cut lettuce irradiated for 30 minutes with the rays of the mercury vapor lamp transmitted through filter A, protected rats completely from rickets when fed in a dose of four grams per day, while four grams of lettuce receiving the same irradiation when intact gave only a partial protection. Thirty minutes irradiation with the mercury vapor lamp through filter A rendered cut alfalfa powerfully antirachitic, whereas the same treatment had no such effect on the intact plant.

As seen from Tables II and III the vitamin D content of both cut lettuce and cut alfalfa irradiated with the mercury vapor lamp for 30 minutes

<sup>2</sup> Filter N is not solarized by sunlight but loses some of its transmission only upon exposure to ultraviolet rays of shorter wave length such as emitted by arc sources.



TABLE I  
EFFECT OF ULTRAVIOLET LIGHT ON THE VITAMIN D CONTENT OF VARIOUS PLANTS

Plant	Light conditions for growth of plant	Irradiation with mercury vapor lamp			Rat growth data							
		Filter used	Length of exposure	Plants rooted or cut	No. of rats in group	Amt. fed per rat (grams)	Av. wt. rat at start (grams)	Av. gain during exp. (grams)	Av. width metaphysis*	Av. ash % of femur	Deviation from ash % of protected rat	Protection from rickets
Spinach	Window Glass	N	None	Cut	5	4	58	+15	0.467	42.7	- 6.6	--
	30 min.		3		4	50	+15	0.450	43.4	- 4.2	--	
	None		3		4	57	+27	0.538	45.1	- 4.0	--	
	None		2		4	60	+34	0.140	52.7	+ 3.1	+++	
New Zealand spinach	Window Glass (in August)		None		3	4	49	+13	0.819	36.7	-10.6	--
	" " " " (in August)		3		6	50	+20	0.514	40.4	- 7.2	--	
	None		3		4	50	+24	0.431	46.7	- 0.9	-	
	None		3		6	51	+21	0.237	47.6	- 0.2	+	
Cabbage	Window Glass	N	None	Cut	5	4	51	+17	0.893	33.0	-14.8	--
	Window Glass (in August)		3		7	60	+21	0.940	32.3	-17.3	--	
	Window Glass (in March)		3		4	47	+15	0.822	37.4	- 8.4	--	
	Filter N (in March)		3		4	49	+ 2	0.615	40.1	- 7.2	--	
	Outside (in August)		3		4	54	+19	0.919	34.3	-14.2	--	
	" " " " (in August)		3		9	60	+25	0.681	37.2	-12.4	--	

TABLE I (Continued)  
EFFECT OF ULTRAVIOLET LIGHT ON THE VITAMIN D CONTENT OF VARIOUS PLANTS

Plant	Light conditions for growth of plant	Irradiation with mercury vapor lamp			Rat growth data							
		Filter used	Length of exposure	Plants rooted or cut	No. of rats in group	Amt. fed per rat (grams)	Av. wt. rat at start (grams)	Av. gain during exp. (grams)	Av. width metaphysis*	Av. ash % of femur	Deviation from ash % of protected rat	Protection from rickets
Swiss Chard	Window Glass (in February)	N	None	Cut	3	4	48	+ 4	0.427	42.2	- 4.2	-
	Window Glass Filter N		30 min.		3	4	46	+ 10	0.103	45.2	- 1.3	+
	(in March)		None		3	4	58	+ 14	0.581	40.2	- 9.1	-
	Outside (in September)		None		3	4	63	+ 26	0.410	43.0	- 7.1	-
Soy Bean	Window Glass Outside	A	None	Rooted† Rooted‡	3	4	54	+ 23	0.558	41.6	- 6.9	-
	(in August)		None		2	4	59	+ 25	0.252	46.9	- 2.5	+
	Outside		None		3	11	65	+ 42	0.342	44.8	- 5.7	-
	(in September)		13 min.		3	4	58	+ 8	0.048	37.7	- 11.6	-
	Window Glass		13 min.		3	4	59	+ 11	0.076	38.0	- 11.4	-
	Window Glass				3	4						-

\* These figures have a comparative value only.

\*\* Very poor spinach, considerably dried by heat.

† Fed immediately after irradiation.

‡ Cut and fed 24 hours after irradiation.

TABLE II  
EFFECT OF ULTRAVIOLET LIGHT ON THE VITAMIN D CONTENT OF LETTUCE

Light conditions for growth of plant	Irradiation with mercury vapor lamp			Rat growth data							
	Filter used	Length of exposure	Plants rooted or cut	No. of rats in group	Amt. fed per rat (grams)	Av. wt. rat at start (grams)	Av. gain during exp. (grams)	Av. width metaphysis*	Av. ash % of femur	Deviation from ash % of protected rat	Protection from rickets
Window Glass		None		15	4	60	+29	0.611	41.1	-8.5	-
Outside		None		5	4	64	+32	0.462	44.7	-5.6	-
Filter N		None		2	15	54	+39	0.424	47.1	-1.4	-
(in winter)		None		7	4	55	+20	0.604	44.5	-4.2	-
Filter N		None		2	12	49	+42	0.278	50.3	+3.0	+
(in summer)	A	1 hr.	Cut	6	4	50	+17	0.212	45.6	-2.0	+
Window Glass	A	1 hr.	Rooted	4	4	57	+20	0.270	45.1	-4.0	+
"	A	30 min.	Cut	4	4	56	+18	0.105	48.6	-0.3	+
"	A	30 min.	Rooted	14	4	55	+12	0.207	45.5	-3.2	+
"	A	13 min.	Cut	8	4	56	+16	0.181	47.2	-1.7	+
"	A	13 min.	Rooted	11	4	55	+16	0.337	43.9	-4.8	+
"	A	13 min.	Rooted†	9	4	55	+14	0.306	43.4	-5.3	-
"	A	7 min.	Rooted	3	4	55	+19	0.523	46.5	-2.2	-
"	A	10 min.									
		per day for 3 days	Rooted	3	4	52	+19	0.482	44.1	-4.0	-
"	N	20 min.	Cut	3	4	48	+14	0.493	40.6	-6.5	-
"	N	10 min.	Cut	3	4	51	+9	0.407	39.9	-7.9	-
"	C	1 1/2 hr.	Rooted	3	4	51	+26	0.384	42.5	-5.3	-
"	C	1 1/2 hr.	Rooted†	3	4	52	+16	0.433	41.9	-6.2	-
"	C	1 1/2 hr.									
		per day for 3 days	Rooted	3	4	52	+22	0.342	48.5	+0.4	-

\* These figures have a comparative value only.

† Cut and fed 24 hours after irradiation.

‡ Cut and fed in 3 days after irradiation.

TABLE III  
EFFECT OF ULTRAVIOLET LIGHT ON VITAMIN D CONTENT OF ALFALFA

Light conditions for growth of plant	Irradiation with mercury vapor lamp			Rat growth data						
	Filter used	Length of exposure	Plants rooted or cut	No. of rats in group	Av. wt. rat at start (grams)	Av. gain during exp. (grams)	Av. width metaphysis*	Av. ash % of femur	Deviation from ash % of protected rat	Protection from rickets
Window Glass		None		10	58	+28	0.753	40.1	-9.2	-
In the open	A	1 hr.		10	62	+31	0.505	44.1	-5.9	-
Window Glass	"	"	Cut	11	57	+19	0.215	46.7	-2.4	+
"	"	30 min.	Rooted	11	57	+17	0.243	46.9	-2.2	+
"	"	"	Cut	11	59	+19	0.163	50.7	+1.3	+
"	"	20 min.	Rooted	4	62	+25	0.929	42.5	-7.5	-
"	"	per day for 3 days	Rooted	6	57	+14	0.190	47.6	-1.5	+
"	"	13 min. per day for 2 days	Rooted	4	62	+28	0.233	49.4	-0.6	+
"	"	10 min. per day for 3 days	Rooted	3	49	+10	0.112	50.9	+3.6	+
"	"	1 hr.	Rooted "†	6	56	+20	0.315	45.6	-3.3	+

\* These figures have a comparative value only.

† Cut and fed in 48 hours after irradiation.

is slightly greater than that of similar plants irradiated for one hour. This phenomenon is in accordance with the fact reported by several investigators (16), that over-irradiation destroys the antirachitic virtue of activated ergosterol. It seems probable that the ergosterol contained in lettuce and alfalfa becomes completely activated after 30 minutes irradiation and that further irradiation destroys its activity. This, however, does not apply to the plants irradiated intact. The ergosterol contained in lettuce and alfalfa does not become completely activated after either 30 or 60 minutes irradiation of the intact plant. The effect of exposing lettuce for 60 minutes was about the same as for 30 minutes, each producing considerable vitamin but neither producing a sufficient amount to protect completely. In the case of alfalfa an exposure of 30 minutes proved to be entirely insufficient, whereas an irradiation of one hour of the intact plant gave fairly good results.

In comparing the efficiency of one long exposure with that of several short exposures of a total equal duration it can be seen (Table III) that three exposures of 20 minutes each, administered one per day, rendered alfalfa more potent antirachitically than one exposure of one hour. Similarly, three exposures of ten minutes each or two exposures of 13 minutes each were more efficient than one exposure of 30 minutes. In the case of alfalfa apparently several short exposures are more efficient in forming vitamin D than one long exposure. However, opposite results were obtained in experiments with lettuce, i.e., three exposures of ten minutes each administered one per day gave considerably less vitamin than one exposure of 30 minutes. These contradictory results make it impossible to reach any definite conclusion which would apply to all plants. Different plants apparently possess individual characteristics which make it impossible to draw any general rules.

TABLE IV  
TRANSMISSION OF FILTERS

Filter	S*	A	B	C	D	F
Thickness in mm.	4.98	1.9	3.11	3.99	2.0	5.06
Wave length in millimicrons						
Transmission 50%	335	299.4	308.8	310.4	310.2	324.2
10%	302	264.9	275.9	286.4	292.3	303.9
1%	265	249.7	259.7	273.7	281.1	293.5
Limit of transmission	253	237	245	253	270	286

\* Transmission data from Coblenz & Stair (4) after exposure to mercury lamp for 75 hours.

From the results reported in Tables II and III it can be concluded that the antirachitic substance formed in the plant with ultraviolet rays is not destroyed by the living plant for at least 24 hours. The antirachitic value

of the irradiated plants fed 24 hours after irradiation was the same as that of the plants fed immediately after irradiation. Plants fed 48 and 72 hours after irradiation seem to have slightly less value. Evidently a living plant retains the antirachitic substances formed by irradiation for about 24 hours, after which time a slow destruction of these substances by the living tissue takes place.

#### EXPOSURE OF ANIMALS TO SUNLIGHT

Rats were exposed to sunlight in the following way: the rats were exposed in a greenhouse covered with filter N six times each week (whether the sun was shining or not). They were kept there during the necessary period of time in wide mesh (one-half inch) screen cages, which did not exclude the sun. As the period of greatest sunlight is between 10:00 A.M. and 2:00 P.M., exposures were made so as to include the brightest part of the day, as follows:

4 hours exposure—from 10:00 A.M. to 2:00 P.M.						
2	"	"	"	11:00	"	1:00
1	"	"	"	11:00	"	12:00
30 min.	"	"	"	11:30	"	12:00
15	"	"	"	11:45	"	12:00

As seen from Table V, young rats on Sherman's diet 84 can be protected from rickets by a short exposure to sunlight transmitted through filter N. During the winter months the minimum protective exposure is about 30 minutes per day; 15 minutes gave an appreciable but not complete protection. During the summer months 15 minutes of exposure to sunlight protects completely. This was the shortest exposure used in this work, and, consequently, it can not be considered the minimum protective exposure for it seems probable that even shorter periods may prove satisfactory.

When the basal diet was changed, as stated above, and lettuce grown under greenhouse glass was given to all the experimental rats fed diet 84, the minimum protective exposure established previously did not always prove satisfactory. For instance, such exposures as one hour per day in March, 30 minutes in May, and 15 minutes in July were too short to produce normal calcification in the bones of the rats receiving four grams of greenhouse lettuce per day. Attention is called to the fact that these rats gained in weight considerably more than those fed only diet 84. This confirms the assumption made previously that the more the rat grows the larger the amount of antirachitic factor needed for normal calcification of the bones.

TABLE V  
DIRECT EXPOSURE OF ANIMALS TO SUNLIGHT TRANSMITTED THROUGH FILTER N

No. of rats in group	Average weight at beginning of experiment (grams)	Average gain in weight (grams)	Month*	Length of exposure	Basal diet	Average width of metaphysis	Average ash of femur	Deviation from ash of protected rat	Protection from rickets
3	50	+6	January	2 hrs.	#84	0.115	51.9	+4.3	++
3	48	+2	"	1 hr.	"	0.122	48.4	+1.3	++
3	53	+2	"	30 min.	"	0.118	49.0	+0.7	"
3	44	0	February	Cont.	"	0.106	44.3	-1.6	"
2	53	-4	"	4 hrs.	"	0.146	49.0	+0.7	"
2	57	-1	"	2 hrs.	"	0.111	46.6	-2.5	"
3	55	+8	"	15 min.	"	0.172	48.4	-0.3	++
3	56	-5	March	Cont.	"	0.125	48.8	-0.1	++
2	57	-6	"	4 hrs.	"	0.094	48.8	+0.3	++
2	58	-3	"	2 hrs.	"	0.097	48.1	-1.2	"
3	55	+18	"	1 hr.	#84 + 4 gms. lettuce Window Glass	0.108	48.7	0	++
3	56	0	April	1 hr.	"	0.135	44.1	-4.8	++
3	53	-6	May	Cont.	#84	0.093	44.0	-4.3	++
3	51	-7	"	4 hrs.	"	0.098	43.4	-3.4	"
3	48	0	"	2 hrs.	"	0.116	44.6	-2.5	"
3	48	-6	"	1 hr.	"	0.084	44.7	-2.4	"
3	56	+12	"	1 hr.	#84 + 4 gms. lettuce Window Glass	0.090	48.6	-0.3	"
4	56	+22	"	30 min.	"	0.224	46.8	-2.1	+
3	56	-9	June	Cont.	#84	0.048	49.2	+0.3	++
3	57	+2	"	4 hrs.	"	0.075	46.3	-2.8	++
3	56	-1	"	2 hrs.	"	0.086	45.8	-3.1	"
3	56	+1	"	1 hr.	"	0.093	46.9	-2.0	"
3	51	0	"	30 min.	"	0.098	45.2	-2.6	"
3	51	-2	"	15 min.	"	0.096	45.2	-2.6	"
2	56	+21	"	30 min.	#84 + 4 gms. lettuce Window Glass	0.137	47.8	-1.1	"

TABLE V (Continued)  
DIRECT EXPOSURE OF ANIMALS TO SUNLIGHT TRANSMITTED THROUGH FILTER N

No. of rats in group	Av. wt. rat at beginning of exp. (grams)	Av. gain in weight (grams)	Month*	Length of exposure	Basal diet	Av. width metaphysis†	Av. ash % of femur	Deviation from ash % of protected rat	Protection from rickets
3	47	+ 2	July	30 min.	#84	0.124	43.5	-3.3	++
3	45	+ 2	"	15 min.	#84 + 4 gms. lettuce	0.113	46.4	+0.2	"
10	74	+ 9	"	1 hr.	Window Glass	0.100	52.3	+0.3	"
9	65	+ 14	"	30 min.	"	0.156	50.6	0	"
4	66	+ 19	"	15 min.	"	0.180	51.0	+0.3	"
5	56	+ 22	August	30 min.	"	0.146	50.0	+1.1	"
3	63	+ 4	September	30 min.	#84	0.104	50.0	-0.1	"
3	67	- 1	"	15 min.	"	0.085	50.0	-0.8	"
6	64	+ 16	"	30 min.	#84 + 4 gms. lettuce	0.138	47.9	-2.4	"
3	53	+ 3	October	1 hr.	Window Glass	0.108	46.1	-2.2	"
3	50	- 2	"	30 min.	#84	0.091	46.4	-1.2	"
3	49	+ 4	"	15 min.	"	0.145	45.3	-2.0	"
3	49	+ 2	November	2 hrs.	"	0.153	46.0	-1.3	"
3	49	+ 5	"	1 hr.	"	0.084	51.3	+4.0	"
3	49	+ 2	"	30 min.	"	0.103	51.1	+3.8	"
3	64	+ 6	December	Cont.	"	0.106	48.9	-1.4	"

\* When an experiment in this table is listed under a certain month, it does not mean that the rats were exposed to sunlight from the first to the last of the month. No attempt was made to start the experiments on the first of the month; they were started when young rats of required weight were available. These experiments were listed under a certain month, the greatest part of which took place during this particular month.

† These figures have a comparative value only.



## EFFECT OF EXPOSING ANIMALS TO MERCURY VAPOR LAMP

In the experiments with direct irradiation of the rats with the rays of the mercury vapor lamp, complete protection was obtained even with filters of comparatively low transmission in the extreme ultraviolet region

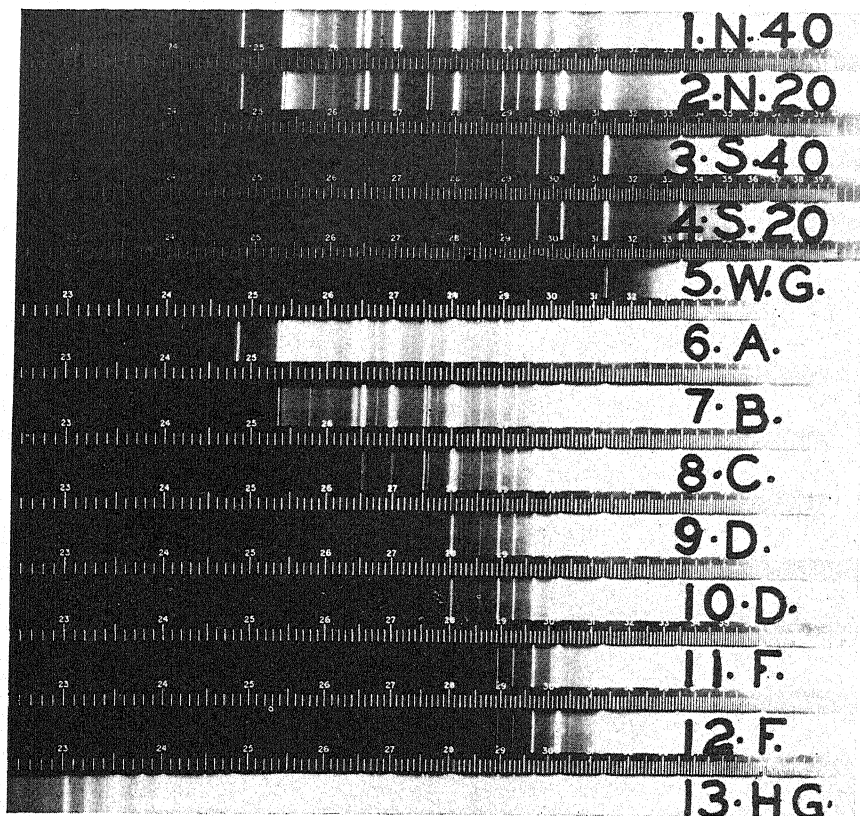


FIGURE 2. Transmission spectra.

1. Filter N before solarization, 40 seconds exposure.
2. Filter N before solarization, 20 seconds exposure.
3. Filter S after solarization, 40 seconds exposure.
4. Filter S after solarization, 20 seconds exposure.
5. Window glass, 20 seconds exposure.
6. Filter, A, 20 seconds exposure.
7. Filter B, 20 seconds exposure.
8. Filter C, 20 seconds exposure.
9. Filter D, 20 seconds exposure.
10. Filter D, 10 seconds exposure.
11. Filter F, 20 seconds exposure.
12. Filter F, 10 seconds exposure.
13. Open arc without filter, 10 seconds exposure.

with very short exposures. The transmission of the various filters used in the work is shown in Table IV. This table with the exception of the column showing the limit of transmission is compiled from data furnished by the Bureau of Standards. A series of spectrograms showing the limits of transmission is reproduced as Figure 2. All data regarding the limits of transmission were obtained at the Institute by means of a Hilger quartz spectrograph. Rats irradiated for one minute per day with the rays of the mercury vapor lamp transmitted by these filters were completely protected from rickets. It will be observed that the most opaque filter F has a transmission limit at wave length  $286\text{ m}\mu$  and that a one minute exposure through this filter is sufficient to protect the animal completely. This indicates that the shorter rays produced by the mercury vapor lamp which are not found in sunlight are of little value in protecting the rats against rickets.

#### DISCUSSION OF RESULTS

The results of this work show that there is an appreciable difference in the antirachitic properties produced by ultraviolet radiation in different green plants. Plants used in this study (lettuce, alfalfa, spinach, New Zealand spinach, soy bean, cabbage, and swiss chard) are found to possess no antirachitic value when grown in the greenhouse. All these plants can be rendered slightly antirachitic by exposure to the extreme ultraviolet region

TABLE VI  
IRRADIATION OF RATS\* WITH THE MERCURY VAPOR LAMP AT A  
DISTANCE OF 15 INCHES

No. of rats in group	Average weight of rat at beginning of exp. (grams)	Average gain in weight (grams)	Filter	Length of exposure, minutes	Average width metaphysis†	Average ash % of femur	Deviation from ash % of protected rat	Protection from rickets
3	49	+1	N	10	Normal bone	47.9	+0.6	+++
3	51	-1	"	5	"	45.8	-2.0	"
3	51	+6	"	3	0.171	46.6	-1.2	++
3	47	+2	B	10	0.104	46.3	-0.5	+++
3	47	+1	"	7	0.093	48.1	+1.3	"
3	51	+2	"	5	0.098	47.4	-0.4	"
2	52	+4	"	3	0.128	47.5	-0.6	"
3	46	+3	"	2	0.112	48.3	+1.8	"
3	46	+1	C	3	0.112	49.0	+2.5	"
3	48	-4	"	2	0.112	47.9	+0.8	"
3	55	+10	D	5	0.110	47.2	-1.5	"
3	55	+8	"	3	0.121	46.6	-2.1	"
3	52	+2	"	2	0.094	46.7	-1.4	"
3	51	0	"	1	0.087	48.5	+0.7	"
3	49	+13	F	5	0.135	49.4	+2.1	"
3	46	+14	"	3	0.139	47.7	+1.2	"
3	50	+5	"	2	0.102	49.9	+2.3	"
3	51	-7	"	1	0.080	48.2	+0.4	"
3	50	+5	None	1	0.099	46.5	-1.1	"

\* All rats were fed diet 84; † These figures have a comparative value only.

of sunlight except cabbage and swiss chard. Of the seven plants listed above, only cabbage could not be rendered appreciably antirachitic by irradiation with the mercury vapor lamp. Alfalfa and lettuce were first in order of antirachitic potency, with spinach, New Zealand spinach, swiss chard, and soy bean following as listed, and cabbage last with practically no potency. Since it is probable that the antirachitic potency of plants depends upon the content of the pro-vitamin ergosterol, which is changed to vitamin D when exposed to ultraviolet radiation, the amount of ergosterol in the different species may be in approximately this same order. That is, alfalfa and lettuce would contain more ergosterol than spinach and soy bean, while cabbage would contain practically none. This assumes that the pro-vitamin is uniformly distributed in the surface cells of all species where it can be reached by ultraviolet radiation.

The minimum daily exposure to ultraviolet rays necessary to protect rats from rickets when irradiated directly is found to be considerably smaller than the minimum exposure of green plant tissue which, when subsequently fed, would protect the rats in a similar manner. An exposure of the animal for one minute daily to the rays from a mercury vapor lamp is found sufficient to produce complete protection, while an exposure of 15 minutes per day to summer sunlight also gives complete protection. On the other hand, the shortest exposure to the mercury vapor lamp which can render plants adequately antirachitic is 30 minutes, and in case of solar radiation even a continuous exposure gives only very slight antirachitic potency to green plants. Since it is believed that in case of direct irradiation of the animal the curative effect of ultraviolet rays is due to the activation of the ergosterol contained in the skin, these results indicate that the content of ergosterol which can be activated by ultraviolet radiation is considerably smaller in plant tissue than in the epidermal layers of animal tissue.

In the study of the effect of direct irradiation of the animal with the rays of the mercury vapor lamp transmitted through various filters it is found that complete protection can be obtained with very short exposures even with filters of low transmission in the extreme ultraviolet region such as filter F (Fig. 2). This filter has a transmission limit at wave length  $286\text{ m}\mu$  and, therefore, absorbs all of the shorter ultraviolet rays from the mercury lamp. It is apparent that the region of shorter wave lengths in the radiation from this lamp has little value in protecting the rat against rickets. Since this filter transmits only one per cent at wave length  $293.5\text{ m}\mu$  the region most effective is seen to lie within the limits of solar radiation, that is, of wave length longer than  $289\text{ m}\mu$ . This agrees with the work of Maughan (21) who found that rays shorter than  $2894\text{ \AA}$  have little curative value on rachitic chickens. Maughan believes that the line  $2968\text{ \AA}$  is the center of the most effective region, while line  $3024\text{ \AA}$  has probably one-

fourth the value of line 2968 Å. Kon, Daniels, and Steenbock (17) have found that activation of ergosterol follows the equivalence law, and 700 to 1000 ergs of radiant energy are necessary for 2560, 2650, 2800, and 2930 Å lines. As pointed out by Maughan, the failure of the shorter wave lengths to cure rickets in the animal is probably due to their failure to reach these cells, which contain the pro-vitamin, ergosterol. Wave lengths longer than 286  $m\mu$  are apparently able to reach the pro-vitamin in the epidermal layers of the animal and in accordance with the observations of Steenbock and others the effectiveness of various lines in this region should be in proportion to their respective energy values.

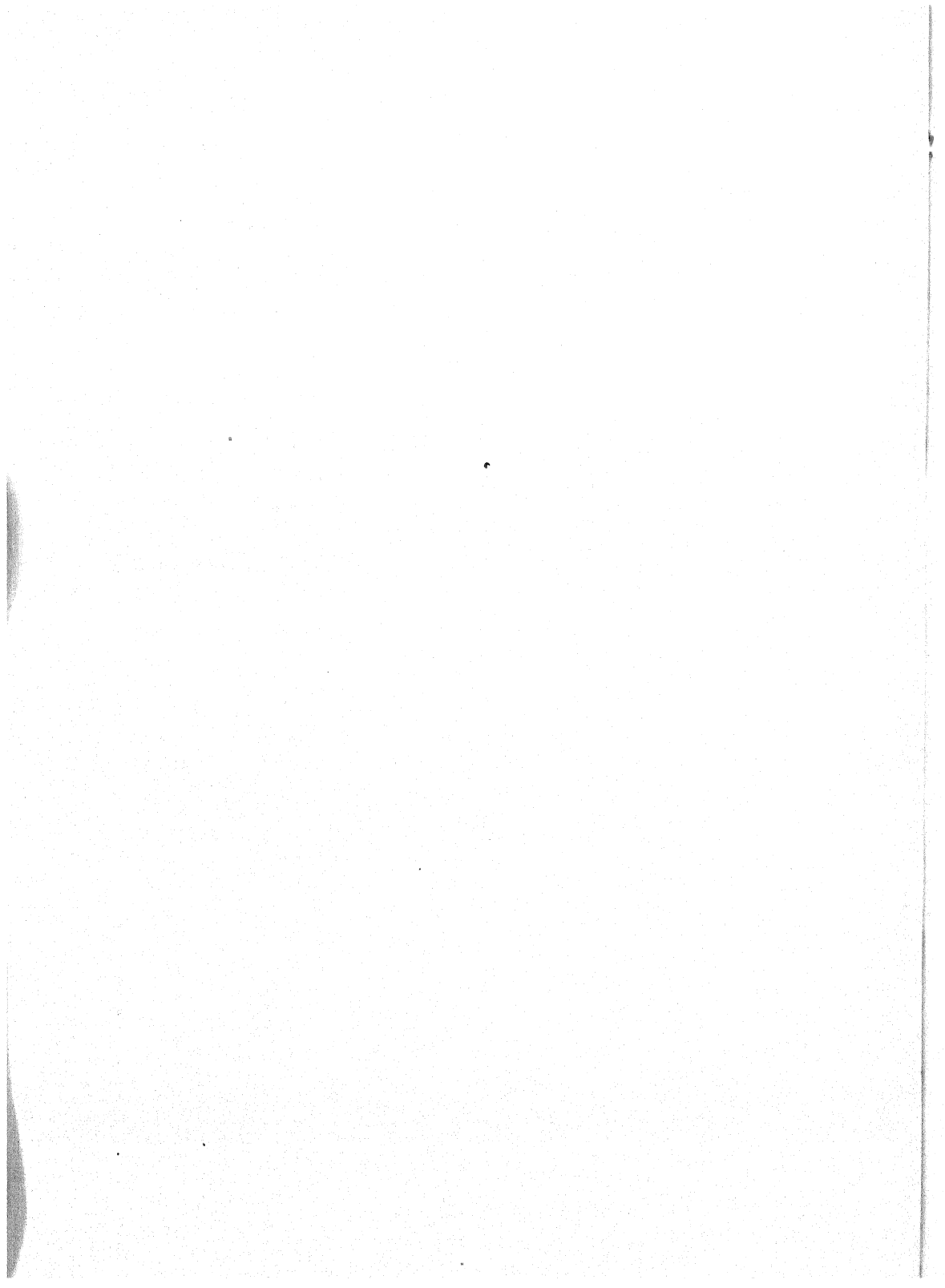
#### SUMMARY

1. Green plants grown in these experiments under ordinary greenhouse glass did not produce vitamin D.
2. Ultraviolet rays from the solar spectrum impart very slight antirachitic value to lettuce, alfalfa, spinach, New Zealand spinach, and soy bean. Rays from the mercury vapor lamp supply these plants with appreciable calcifying properties. No antirachitic properties were produced in cabbage by ultraviolet rays from either source.
3. Irradiation with the rays of the mercury vapor lamp imparts measurably greater antirachitic properties to the cut plant than to the intact plant.
4. The antirachitic substance formed in the living plant by ultraviolet radiation is not destroyed for at least 24 hours.
5. Complete calcification of the bones takes place when young rats on Sherman's diet 84 are exposed to sunlight transmitted through filter N for a period of 30 minutes in winter or 15 minutes during the summer and spring. The addition of lettuce grown under window glass to diet 84 produces an increase in the growth of young rats. For this reason longer exposures to ultraviolet rays are necessary to obtain complete protection against rickets when such green plant tissue is fed.
6. An exposure of one minute per day to the rays of a mercury vapor lamp transmitted through various filters used in these experiments gave complete protection to rats on Sherman's rickets-producing diet 84. Wave lengths shorter than 285  $m\mu$  had little or no protective value on rats. The region most effective in the cure of rickets in rats was found to be within the limits of solar radiation, that is, of wave lengths longer than 290  $m\mu$ .
7. The minimum exposure to ultraviolet rays necessary to produce an appreciable amount of vitamin D in plant tissue is considerably longer than that required to impart complete protection in case of direct irradiation of the animal.

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# THE pH AND THE PHOSPHORUS CONTENT OF THE EXPRESSED LIQUIDS FROM SOILS AND PLANT TISSUES

M. M. MCCOOL AND W. J. YODEN

## INTRODUCTION

It occurred to us during our studies on various peats and mucks that the removal of liquid from them by the application of different pressures might yield facts of interest and importance. Accordingly an outfit was perfected and put into use. The cylinder and plunger of carbon steel are so made that the liquid removed by pressure is forced out at the bottom through fine grooves cut in the bottom of the former and the base upon which it rests. By the use of a hydraulic press and this apparatus we found the pH of the liquid expressed from soils and certain plant tissues to become greater as the pressure was increased. The phosphorus content of the liquid removed from soils in this manner became less as the pressure was increased.

## REVIEW OF LITERATURE

Several successful attempts have been made to remove liquid from soils by the application of pressure. The early literature was reviewed by Morgan (6) and Burgess (2). Lipman (4) placed soils in a special cylinder and applied a maximum pressure of 53,000 pounds per square inch. About 75 per cent of the 15 per cent water in sandy loam soil was removed by this method. He was able to obtain ample liquid from a clay soil whose moisture content was 20 per cent, for density and other determinations. Specimens for study could be obtained from peat carrying a water content of 40 per cent. He does not report, however, data resulting from investigations of the expressed liquid.

Burgess (2), utilizing the Lipman method in his studies on the soil solution, varied the pressure applied to Yolo sandy loam of what he termed optimum moisture content. The fraction obtained from 0 to 1600 pounds pressure per square inch showed within experimental error the same specific gravity as that obtained by the use of a pressure range from 1600 to 5730 pounds. The total soluble solids, inorganic soluble solids, and organic soluble solids present in solutions obtained from a given soil by employing the above pressures varied but slightly, although the fluid obtained by a given range in pressure from different soils showed a rather wide range in these properties. Similar results were recorded with respect to the specific resistance and also to the Ca and Mg contents. On page 199 (2) he states, "These data, . . . , would seem to prove that, with fine sandy loams at least, the application of direct pressures up to approximately 16,000



pounds per square inch has little, if any, effect on the quantitative composition of the expressed solutions, and suggest that the concentration of the part of the soil water which remained in the soil probably carried similar amounts of solutes at the time of extraction."

McCool and Millar (5) compared the freezing point lowerings of triturated tops and roots of corn plants and the expressed sap of the same after having been frozen. The pressure applied was 300 kilograms per square centimeter. The results obtained were similar except for the root studies in which case there was a tendency for the expressed sap to be of lower density than the tissue, especially when the freezing point depressions were great. Reference was made to the possibility of the removal of materials from solution by adsorption.

Knudson and Ginsburg (3) found the density of the sap pressed from differently treated leaves to be greater when subjected to a pressure of 3908 pounds per square inch than that which was obtained when exposed to a pressure of 781 pounds per square inch and suggest the advisability of recording the pressures employed in such studies.

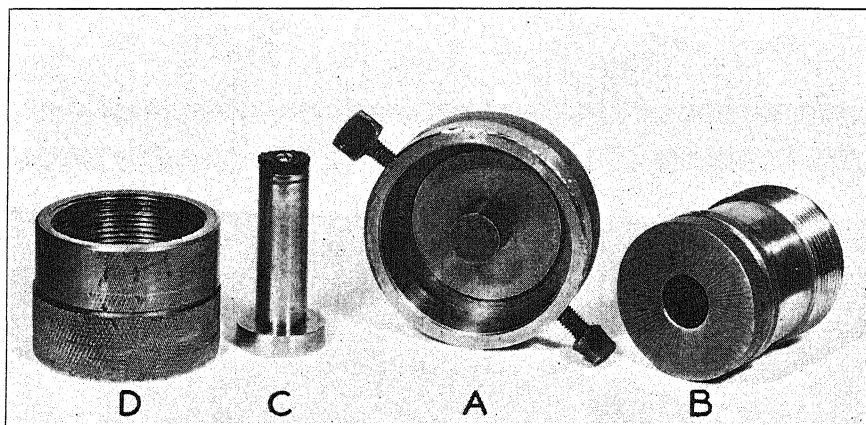


FIGURE 1. Device for expressing liquid from materials through the application of pressure. A, base; B, cylinder; C, plunger; D, cap to assist in removal of plunger.

Since this paper was written Sayre and Morris (7) have reported the total solids content of sap pressed from the stems and leaves of corn under a pressure of 100 pounds per square inch to be less in successive portions. The sucrose and free reducing sugars in successive portions of sap removed from ground materials were constant, but less in case of minced tissues.

## EXPERIMENTAL RESULTS

## THE PH OF LIQUID REMOVED FROM PEATS AT DIFFERENT PRESSURES

The pH of liquid expressed from a brown, partially-decomposed, sphagnum peat from Germany, a dark brown mainly sedge peat, and a black aquatic peat collected in the vicinity of Fishkill, New York, was determined by the glass electrode and also by indicators. Twenty-five gram samples were placed in a hand screw meat press, samples of the expressed liquid taken for study, and the solid portion transferred to the cylinder shown in Figure 1. This was, in turn, placed in position in the hydraulic press and exposed to different pressures. The cylinder remained in position five minutes under each of the pressures recorded. The liquid forced from the samples was collected in weighing bottles and the pH readings taken immediately. The amount of water removed from hand pressed samples of the brown sphagnum peat and the fibrous or sedge peat by different pressures was determined. In these determinations 20 grams of the former containing 68.6 per cent moisture and 50 grams of the latter containing 64.5 per cent moisture were used. As the data in Table I bring out, the

TABLE I  
LIQUID REMOVED FROM HAND PRESSED PEATS BY DIFFERENT PRESSURES

Pressure, lbs. per sq. inch	Sphagnum peat, cc. H <sub>2</sub> O	Sedge peat, cc. H <sub>2</sub> O
1,000	5.4	7.2
2,000	1.9	1.2
4,000	0.5	0.4
10,000	0.2	0.4
20,000	0.1	0.1
60,000	0.05	0.05

greater part of the water pressed from these was derived from the application of 1000 pounds pressure to the square inch. Owing to the small quantity obtained at the higher pressures a pipette was employed to remove the liquid from the base upon which the cylinder rests and when necessary several samples were brought together to form a composite for study. It was found advisable to detach the cylinder from its base and remove the moisture from the various parts with a clean dry cloth after each pressing was completed. The glass electrode was found to be very suitable for the pH determinations inasmuch as a small drop of the liquid is ample.

Before subjecting the peat to pressure samples were shaken with water, or the desired salt solution, in the proportion of one gram of material to ten cc. of liquid. After standing overnight, the excess liquid was removed by decantation and portions of the residues subjected to the pressures indicated in Tables II, III, and IV. Table II shows the results of seven ex-

periments, two with water, two with calcium chloride solutions at concentrations of M/50 and M/10, and three with M/100 monobasic calcium phosphate solution. It is to be noted that the liquid obtained by the hand press from the variously treated peat samples had a low pH. The applica-

TABLE II

THE PH OF LIQUID REMOVED FROM BROWN SPHAGNUM PEAT BY DIFFERENT PRESSURES

Pressure, lbs. per sq. inch	Untreated		CaCl <sub>2</sub>		Ca(H <sub>2</sub> PO <sub>4</sub> ) <sub>2</sub> M/100		
			M/50	M/10			
Hand	3.57	3.64	3.30	2.81	3.23	3.27	3.15
1,000	4.85	5.65	5.62		4.59	4.06	4.76
2,000		5.99		4.79			
3,000	5.37	6.40	5.96		5.43	4.76	5.37
5,000	6.19	6.74	6.30	6.18	6.94		5.97
10,000	6.36	6.62	6.36				
30,000	6.41	6.77	6.24	6.08		5.08	

tion of a pressure of 1000 pounds per square inch to the hand pressed samples, however, resulted in great changes in this respect. Further changes were obtained with increased pressures, but these as a rule were slight with pressures exceeding 3000 pounds per square inch.

TABLE III

THE PH OF LIQUID REMOVED FROM BLACK PEAT BY DIFFERENT PRESSURES

Pressure, lbs. per sq. inch	Untreated		CaCl <sub>2</sub> M/50	
Hand	4.96	4.55	4.77	4.65
1,000	5.84	5.42		
2,000	6.09	6.04		
3,000	6.52	6.36	5.33	5.83
4,000	6.77			6.18
5,000	6.70			
6,000	6.70	6.50		6.41
8,000	6.35			
10,000			6.50	6.37
30,000	6.45	6.36		6.41

According to the data in Tables III and IV similar relationships were found to exist between pressure and the finely divided aquatic peat, and also the more fibrous or sedge peat. The use of salt solutions increased the acidity of the liquids expressed at low pressures. At high pressures the liquid obtained appeared not to be influenced by the salt treatment.

#### THE PH OF SAP PRESSED FROM PLANT TISSUES

The pH of juice pressed from *Coleus blumei* Benth. and tomato (*Lycopersicum esculentum* Mill.) stems and petioles and last year's growth Jonathan apple (*Pyrus malus* L.) twigs was ascertained. These portions of the

plants were used because of the difficulty in preventing the pulp of the leaves, or less fibrous materials, from being forced out of the cylinder. The samples were ground in an ordinary food grinder before they were subjected

TABLE IV  
THE pH OF LIQUID REMOVED FROM BROWN FIBROUS PEAT BY DIFFERENT PRESSURES

Pressure, lbs. per sq. inch	Untreated		Ca(H <sub>2</sub> PO <sub>4</sub> ) <sub>2</sub> M/100	
Hand	5.97	5.84		
1,000	6.76	5.83	5.57	5.61
2,000	7.04	6.04		5.83
3,000	6.87	6.14	6.46	6.14
4,000				
5,000		6.14	6.50	6.31
6,000				
8,000				
10,000	6.70	6.41		6.71
30,000	6.82	6.70	6.82	6.64

to the various pressures. Some of the data resulting from these studies are presented in Table V. Sample A of the coleus was taken two weeks earlier than sample B which was in bloom at the time of sampling. According to these results similar relationships exist between the pH of the hand expressed juice from plant tissues, and that by different pressures, as main-

TABLE V  
THE pH OF SAP PRESSED FROM PLANT TISSUES

Pressure, lbs. per sq. inch	Coleus stems		Tomato stems		Apple twigs	
	A	B	A	B	A	B
Hand	5.84	5.8	5.52	5.6	3.91	3.98
1,000	5.82		5.65	5.76		4.70
2,000	5.91				4.84	
3,000	6.14	6.2	5.93	6.09		5.12
5,000	6.30	6.6	6.01	6.15		
10,000	6.30	6.8		6.80	5.87	5.64
30,000	6.30		6.80	6.80		6.24

tained with the peats. In this respect the most rapid change in the pH was noted in the case of the apple twigs. The average pH of the juice removed by the hand press was 3.94, the pH of that removed from sample B by the 1000 pound pressure was 4.70, at 5000 pound pressure it was 5.12, and at 30,000 it was 6.24.

#### THE PHOSPHORUS CONTENT OF MOISTURE REMOVED FROM SOILS

The phosphorus contained in water removed from soils by the application of different pressures to them was ascertained by means of the method described by Spurway (8). The standard phosphate solutions were pre-

pared as directed by Truog (9). Representative data which were obtained are given in Tables VI and VII.

TABLE VI  
THE PHOSPHORUS CONTENT OF LIQUID REMOVED FROM BROWN SPHAGNUM  
PEAT BY DIFFERENT PRESSURES; P. P. M.

Pressure, lbs. per sq. inch	Untreated		CaCl <sub>2</sub> M/50
	A	B	C
Hand	50	55	4
1,000	50	50	1
4,000	15	12	0.5
10,000	6	6	Trace
20,000	4	3	Trace
30,000	4	4	Trace

TABLE VII  
THE PHOSPHORUS CONTENT OF LIQUID REMOVED FROM BROWN FIBROUS PEAT AND  
SANDY LOAM SOIL BY VARIOUS PRESSURES; P. P. M.

Pressure, lbs. per sq. inch	Fibrous peat			Sandy loam	
	Ca(H <sub>2</sub> PO <sub>4</sub> ) <sub>2</sub>			No treatment	
	A	B	C	A	B
Hand	3	4	40		
1,000	3	3	30	4	5
2,000	3	2	2.5	3	2
4,000	2	2	2.5	0.5	1
10,000	0.5	0.5	1	0.25	0.25
30,000	0.5	0.5	0.5	Trace	0.25

According to these results samples A and B of the sphagnum peat carried a relatively large amount of phosphorus in solution. Sample C which had only a few parts of phosphorus per million in solution was treated with M/50 CaCl<sub>2</sub>. The liquid obtained from samples A and B by means of the hand press contained 50 parts per million of phosphorus and that removed by the 4000 pound pressure 15 and 12 respectively. When subjected to a pressure of 30,000 pounds per square inch the liquid obtained carried four parts per million of phosphorus. The phosphorus in the liquid derived by means of the hand press from the calcium treated sample, or C, decreased from four parts to one-half part per million in that obtained by the application of 2000 pounds pressure per square inch. Lesser amounts were noted in that obtained by the use of the greater pressures.

Samples A and B of the brown fibrous peat were brought into contact with a solution of monobasic calcium phosphate containing 12 parts per million phosphorus and sample C with one carrying 50 parts per million. An examination of the data in Table VII shows that the liquid obtained by means of the hand press from sample A contained the same amount of

phosphorus as that forced out by 1000 and 2000 pounds pressure but there was less phosphorus in the fluid obtained by means of the great pressures. The amount of phosphorus in the liquid removed by means of the hand press from samples B and C was greater than that in the liquid forced out by the higher pressures, there being very little in the final specimens. The phosphorus in the water removed by each succeeding pressure from the sandy loam soil decreased until there was only a trace present in that obtained by the application of 10,000 and 30,000 pound pressures respectively.

#### EFFECT OF MOISTENING AND REPRESSING

The final pressing at 30,000 pounds per square inch of brown sphagnum peat gave a liquid whose pH ranged from 6.41 to 6.77 in different runs. The hydrogen ion concentration thus decreased several hundred-fold from the first liquid obtained with the hand press, the pH of this fraction being close to 3.6. The peat cake, after this high pressure treatment, was broken up, remoistened, and let stand for 14 hours. The fraction obtained by means of the hand press then had a pH of 3.91 showing a return to substantially the same acidity initially shown. Other samples of the brown sphagnum peat, as well as those of the brown somewhat fibrous peat, gave similar results. Like relationships were found to exist with respect to phosphorus. A sample of the brown sphagnum peat was hand pressed and the phosphorus content of the liquid removed was found to be 50 parts per million. The same material was subjected to 30,000 pounds pressure, remoistened, and again hand pressed. The phosphorus content of the moisture so derived was found to be 35 parts per million. The brown somewhat fibrous peat reacted in a similar manner. It should be noted also that there remains considerable moisture in the materials after having been under 30,000 pounds pressure per square inch. The brown sphagnum peat, for example, retained 44.4, the black finely divided peat 47.4, the sandy loam 7.4, the coleus 66.1, and the tomato 72.4 per cent moisture respectively.

#### DISCUSSION

The lowered hydrogen ion concentration and phosphorus content of the fractions obtained from peats by the application of increasing pressures does not imply the exhaustion of the acidity reserve or the phosphorus content of these materials. On rewetting these materials give fractions similar to those initially observed as far as the hydrogen ion concentration and phosphorus content are concerned. Since living plant tissues also gave fractions of greatly decreased acidity under high pressures it would indicate that caution must be observed in drawing conclusions about the original material from samples of liquids forced out by pressure. Liquids obtained with pressures as low as 1000 pounds per square inch show great

changes over the fraction readily extracted with a hand press. The materials vary in the pressures required to procure the maximum changes observed. Frequently the fractions showed little additional change on increasing the pressure from 3,000 pounds to 30,000 pounds which was the maximum employed.

These phenomena may be due to the solid particles in the samples which may act as a series of semi-permeable membranes and under pressure release the liquid but hold back materials previously in solution. Bouyoucos (1) has pointed out that there may be a less concentrated solution in immediate contact with the surface of the particles and in the very fine capillary spaces, and supposes that the water is held in such a manner that it does not function in its usual capacity as a solvent.

Certain phases of this problem are being investigated and later data may make possible more definite statements in attempting to explain the results obtained.

#### SUMMARY

This is a preliminary report on the differences observed in the liquids removed from soils by the application of various pressures up to 30,000 pounds per square inch. We have found by means of a hand screw meat press and a hydraulic press that the acidity, as determined by the pH readings of liquid removed from soils, from the stems of coleus, tomato, and apple twigs by the application of different pressures, becomes decidedly less as the pressure is increased. In this respect marked changes were noted even when the hand pressed samples were subjected to 1000 pounds pressure per square inch. Further reductions took place as the pressure was increased.

The phosphorus content of the moisture removed from soils became less as the pressure was increased. There was appreciably less phosphorus in the liquids derived from the samples by the application of 4000 pounds per square inch to them than there was in the liquid obtained by means of the hand press. The solutions obtained by means of the very high pressures in several instances contained less phosphorus than one-half part per million. Upon rewetting pressed peat specimens and hand pressing several hours later the hydrogen ion concentration and the phosphorus in the liquid thus obtained were nearly as great as they were in the liquid obtained by the first hand pressing.

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# THE EFFECT OF THIOCYANATES UPON AMYLASE ACTIVITY.

## I. POTATO AMYLASE<sup>1</sup>

F. E. DENNY

### INTRODUCTION

Previous experiments (3, 4) have shown that when freshly-harvested potato (*Solanum tuberosum* L.) tubers are cut into pieces and soaked in solutions of thiocyanates, the rest period is broken and prompt sprouting of dormant buds begins.

It was shown further (5) that favorable treatments for inducing sprouting also caused a decrease in the starch content, and, since the enzyme amylase is involved in starch hydrolysis, the question at once arises: Does the thiocyanate that is absorbed by the tissue act in a direct way by hastening amylase action, and does sprouting begin when this amylase is stimulated into increased activity?

Johnson and Wormald (8) have suggested that perhaps this is the case; after finding that small amounts of potassium thiocyanate hastened the amylase of saliva, they carried out similar tests with plant diastases using malt and also a potato amylase which had been prepared from dialyzed potato juice; in both cases they found a stimulating effect, KSCN producing a noticeable acceleration, at least in the early stages of starch hydrolysis.

Experiments were undertaken to test the direct effect of thiocyanates upon these two types of amylases. Section I (the present paper) deals with potato amylase, and Section II (the paper immediately following) with salivary amylase.

The experiments with potato amylase take into account the question whether a stimulating effect can be observed in press-juice or only in dialyzed juice, and whether the amount of thiocyanate usually absorbed by the tissue in the chemical treatments (not more than 10 mg. of NaSCN per 100 cc. of press-juice) is sufficient to bring about any changes in the amylase activity of the juice.

### METHODS

Two methods of determining amylase activity were used: (a) by measuring the amount of reducing sugar formed by the action of the potato juice upon soluble starch; this is a measure of the *saccharogenic* activity; (b) by adding iodine and noting the effect of the juice in breaking down starch into substances that no longer give a blue color with iodine; this is a measure of the *amylolytic* activity.

<sup>1</sup> Herman Frasch Foundation for Research in Agricultural Chemistry, Paper No. 11.

*Saccharogenic activity.* The mixture of potato juice, buffer, starch, and water or thiocyanate solution was placed in 100 cc. Pyrex test tubes and was rotated in a constant temperature oven. But, corresponding to each lot containing starch, there was a check lot containing water instead of starch solution; in this way any increase in copper-reducing power due to autolytic changes in the check lot containing no starch was deducted from the increase due to the action of the amylase upon the soluble starch. The difference between the potassium permanganate ( $\text{KMnO}_4$ ) titration values of these two lots, one in the presence and the other in the absence of soluble starch, was regarded as a measure of the saccharogenic activity of the sample of juice. In estimating the reducing sugar, the Munson and Walker (1, p. 78) procedure was followed except that the cuprous oxide was titrated with a potassium permanganate solution standardized with dextrose rather than with copper. Each cc. of the  $\text{KMnO}_4$  solution was equivalent to 1.58 mg. of dextrose.

*Amylolytic activity.* The mixture of juice, buffer, Lintner's soluble starch, and water or thiocyanate solution was tested with iodine at the end of 20 to 44 hours for the disappearance of the blue color with starch. In making the test for the presence of starch with iodine, it was not possible to follow the simple procedure usually recommended, i.e., merely to add iodine and note the color. During the prolonged period of rotation of the reaction-mixtures in the oven the juices became so dark colored that the starch test could not be applied unless the liquid was diluted; but this dilution was undesirable since it lowered the concentration of any starch that was present. This difficulty was overcome in the following way: a sample of the liquid in a test tube was placed in a boiling-water bath for 5 minutes; it was then cooled and 1 cc. of  $\text{HCl}$  (sp. gr. 1.18) for each 10 cc. of liquid was added; after filtration the liquid obtained was yellowish but fairly clear even from a juice which was originally very dark. Absence of the blue color upon the addition of iodine in potassium iodide solution was the end-point that was used as a measure of amylolytic activity. In adding the iodine it is essential that the iodine-potassium iodide solution be of such dilution that several drops are needed to bring out the full color; if the iodine concentration is too high the end-point may be passed by, and the test may be negative even in a solution that gives a distinct blue color by careful addition of dilute iodine.

In order to take into account any effect which the added thiocyanate could have upon the colors that were obtained when the iodine was added, it was found desirable to equalize the amounts of thiocyanate in all lots just before the test was applied. In any given series, therefore, enough thiocyanate was added to each lot to make the total concentration the same as that in the lot to which the largest amount of thiocyanate had been added at the start of the experiment. Thus, variable amounts of thiocya-

nate were in contact with the juice during the period in which the amylase was acting, but equal amounts were present when the final test was made. This precaution is of no importance for the sugar determinations for the reason that the amounts of thiocyanate added do not influence the amount of copper reduced; but in the iodine tests for the disappearance of the blue color with starch this factor may become of critical importance for the reason that thiocyanate reacts with iodine and may modify the color formed in the mixture; for example, if the solution is not acidified strongly previous to the addition of the iodine, and if an insufficient amount of iodine is added, the iodine will react with the thiocyanate rather than with the starch, and the starch test will be negative even when starch is present.

*Precautions.* In carrying out the tests by either the saccharogenic or amylolytic method certain precautions are necessary: all glassware and all corks coming in contact with the juice must be heated in boiling water or cleaned with cleaning fluid; the starch, buffer, or added salt solutions must be boiled before the juice is added; chemicals if added in the dry condition should be sterilized first by heat; toluene must be added in order to keep the mixture sterile.

The need of such care in avoiding contamination in amylase experiments has been emphasized by Takane (13) and by Malyshev (10), and is necessary particularly in experiments with potato amylase because the activity of potato amylase is so weak. Thus, in our experiments it was found that the amylase of saliva is at least 20,000 times as active as that of the press-juice of potato. Consequently, the avoidance of contact with saliva is very important. A common source of error consists in pipetting solutions by mouth suction, or by blowing directly into pipettes. It was found necessary to place a bent-tube adapter on all pipettes so that at no time was it possible for even a trace of saliva to get into a pipette.

Another factor that needed to be taken into account is that mentioned by McGuire and Falk (9) who show that the effect of an added salt may be not to increase amylase activity but merely to precipitate the starch. They explain the results of Doby (6) on the activating effect of sodium fluoride on potato amylase as being caused by precipitation of starch by NaF which caused the starch test to appear to be negative when in fact starch was present in the precipitate. The disturbing influence of precipitation in estimating amylase activity even in the absence of added salts was emphasized by Starkenstein (12); he found it desirable to rotate the reaction tubes continuously in order to keep the starch and the amylase in constant contact. For the present experiments the Starkenstein procedure was adopted and all the tubes were rotated slowly, end over end, on a turning bar inside an oven whose temperature was regulated at 35° C.

*Buffering.* Different pH values were maintained by the use of Sorensen's phosphate mixtures as described by Clark (2). Potato juice as pressed

usually has a pH value of about 6.0 to 6.4 and in these experiments the range from 6.0 to 7.3 was tested. In any experiment, if only one pH value was used, the one selected was about 6.2. In order to adjust the pH of the juice to that of the buffer at the pH at which it was desired to carry out the test, dilute NaOH was added to the juice, in each case adjusting the concentration of the NaOH in such manner that 5 cc. of the alkali would just bring 20 cc. of juice to the proper pH; a chart prepared by preliminary tests permitted all subsequent adjustments of pH to be made quickly.

*Varieties.* The main experiments were carried out with juice from the variety Early Ohio; but after the principal effects were established tests were also carried out with juices from the varieties Irish Cobbler and Bliss Triumph; it was found that the different varieties behaved in a similar manner in all essential respects.

*Starch.* For the saccharogenic tests either commercial soluble starch or Lintner's soluble starch made from potato starch according to the directions given by Plimmer (11) were used; but for the amylolytic tests the Lintner preparation was found to be preferable.

## EXPERIMENTAL RESULTS

### EFFECT OF THIOCYANATE UPON THE SACCHAROGENIC ACTIVITY OF POTATO AMYLASE

*With press-juice.* Table I shows the effect of various amounts of sodium thiocyanate upon the amylase activity of potato amylase. The reaction mixture consisted of 30 cc. of the diluted juice adjusted to the proper pH, 30 cc. of 0.2 M phosphate buffer containing the required amounts of sodium thiocyanate to give the concentrations shown in column 1, and 30 cc. of water or of 3 per cent soluble starch. After rotation in the constant temperature oven for 42 hours the reaction mixture was added to boiling alco-

TABLE I  
EFFECT OF SODIUM THIOCYANATE UPON THE SACCHAROGENIC ACTIVITY OF POTATO AMYLASE

NaSCN added per 100 cc. of reaction- mixture (mg.)	Amylase activity expressed as cc. KMnO <sub>4</sub>		
	pH 6.0	pH 6.4	pH 6.8
None	11.9	13.6	15.8
10	10.0	13.0	14.0
30	9.4	11.6	13.0
90	8.6	10.2	11.1
270	6.6	7.5	5.6

hol to give a final concentration of 70 per cent alcohol (by volume); this was boiled, cooled, made up to the mark in a volumetric flask, filtered,

and, after an aliquot was taken the alcohol was evaporated and replaced by water; aliquots of the aqueous solutions were taken for the sugar determinations. The values shown in columns 2, 3, and 4 in Table I are the differences in the permanganate titrations of the lots containing starch and the corresponding checks containing no starch. The figures represent the amylase activity of an aliquot corresponding to 2.6 cc. of the undiluted press-juice. Early Ohio variety was used.

Table I shows that amounts of NaSCN in excess of 10 mg. per 100 cc. of reaction-mixture retarded the saccharogenic activity of potato amylase; at 270 mg. the values were reduced to nearly 50 per cent of the value of the check; at 10 mg. the differences were small and possibly not significant.

Table II shows the result of a similar test in which potassium thiocyanate instead of the sodium salt was added; the starch added as substrate in this test was Lintner's soluble starch. A result similar to that with

TABLE II  
EFFECT OF POTASSIUM THIOCYANATE UPON THE SACCHAROGENIC ACTIVITY  
OF POTATO AMYLASE

KSCN added per 100 cc. of reaction- mixture (mg.)	Amylase activity expressed as cc. $\text{KMnO}_4$		
	pH 6.22	pH 6.68	pH 7.32
None	15.2	21.6	8.8
10	13.8	20.9	9.6
30	10.5	12.5	8.4
90	9.1	9.4	8.5
270	7.5	7.8	5.3

NaSCN was obtained, the thiocyanate causing a depression in amylase activity especially at the lower pH values; at pH 7.32, however, the addition of KSCN had little effect upon the amylase until 270 mg. per 100 cc. had been added.

In other experiments the range from 0 to 10 mg. of NaSCN per 100 cc. of reaction-mixture was tested, but no differences in the amylase activity could be found.

*With dialyzed juice.* Juice from Early Ohio potatoes was dialyzed in colloidion bags for 6 hours in tap water and for 16 hours in distilled water. The addition of 10 mg. KSCN per 100 cc. of reaction-mixture depressed the amylase value from 13.3 cc. of  $\text{KMnO}_4$  to 8.6 cc.; and 50 mg. of KSCN reduced it still further to 4.6 cc.; these values are for an aliquot equivalent to 4 cc. of the potato juice before dialysis. No extensive experiments on the effect of KSCN on the saccharogenic activity of dialyzed juice were carried out for the reason that it seems unlikely that results with dialyzed juice

have much significance in relation to the chemical treatment of potato tubers.

# EFFECT OF THIOCYANATE UPON THE AMYLOLYTIC ACTIVITY OF POTATO AMYLASE

*With press-juice.* The effect of added thiocyanate upon the ability of the amylase of potato juice to hydrolyze Lintner's soluble starch to the stage at which the blue color is no longer given upon adding iodine is shown in Table III.

TABLE III  
EFFECT OF THIOCYANATES UPON THE AMYLOLYTIC ACTIVITY OF POTATO JUICE

Soluble starch added per 25 cc. of reaction-mixture (cc.)	Color obtained upon addition of iodine							
	Amount of KSCN per 100 cc. of reaction-mixture				Amount of NaSCN per 100 cc. of reaction-mixture			
	None	6 mg.	24 mg.	96 mg.	None	6 mg.	24 mg.	96 mg.
	None	6 mg.	24 mg.	96 mg.	None	6 mg.	24 mg.	96 mg.
4.0	Blue	Blue	Blue	Blue	Blue	Blue	Blue	Blue
3.5	"	"	"	"	"	"	"	"
3.0	"	"	"	"	"	"	"	"
2.5	Bluish	Bluish	"	"	Bluish	Bluish	Bluish	"
2.0	Yellow	Yellow	Bluish	"	Yellow	Yellow	Yellow	"
1.5	"	"	Yellow	Bluish	"	"	"	Bluish
1.0	"	"	"	Yellow	"	"	"	Yellow

Into test tubes of 30 cc. capacity were placed 5 cc. of potato juice which had been brought to pH 6.2 by the addition of the proper amount of alkali, 5 cc. of 0.2 M phosphate buffer at pH 6.2, 5 cc. of water or thiocyanate solution, and 10 cc. of Lintner's soluble starch of the proper concentration to give the amounts of 1 per cent starch shown in column 1, Table III; the amounts of added thiocyanate are given at the tops of columns 2 to 9. The body of the table shows the colors obtained when the iodine test was applied according to the procedure described under the heading "Methods." It is seen that very little effect of added thiocyanate was noted. In fact, not until the amount of thiocyanate added reached 96 mg. per 100 cc. of reaction-mixture was an appreciable retardation shown; the slightly retarding effect of 24 mg. in column 4 was not substantiated by the results in column 8.

Tests were made in the range from 0 to 10 mg. of NaSCN per 100 cc. of reaction-mixtures but no effect of the added thiocyanate upon the amylolytic activity was noted.

*With dialyzed juice.* The results with potato juice that had been dialyzed in running tap water for 24 hours are shown in Tables IV and V. The juice was dialyzed in running tap water in bags of collodion made from a mix-

ture of 5 grams Union Cotton Negative, 50 cc. of ethyl ether, and 50 cc. of ethyl alcohol. During dialysis the volume of liquid approximately doubled. The procedure in making the test of amylolytic activity was the same as that described for press-juice, except as to the amount of juice and starch

TABLE IV  
EFFECT OF SODIUM THIOCYANATE UPON THE AMYLOLYTIC ACTIVITY OF DIALYZED POTATO JUICE

Soluble starch added per 25 cc. of reaction-mixture (cc.)	Color obtained upon addition of iodine							
	Dialyzed juice, Bliss Triumph variety				Dialyzed juice, Irish Cobbler variety			
	Amount of NaSCN per 100 cc. of reaction-mixture				Amount of NaSCN per 100 cc. of reaction-mixture			
	None	6 mg.	24 mg.	96 mg.	None	6 mg.	24 mg.	96 mg.
4.17	Blue	Blue	Blue	Blue	Blue	Blue	Blue	Blue
2.46	"	"	"	"	"	"	"	"
1.45	Yellow	Yellow	Yellow	Bluish Yellow	Yellow	Yellow	Bluish	"
0.85	"	"	"	"	"	"	Yellow	Bluish
0.50							Yellow	Yellow

used. In the experiment described in Table IV the amount of dialyzed juice was kept constant at 10 cc. in a 25 cc. reaction-mixture and the amount of starch was varied; in Table V the amount of starch was kept

TABLE V  
EFFECT OF SODIUM THIOCYANATE UPON THE AMYLOLYTIC ACTIVITY OF DIALYZED POTATO JUICE

Amount of dialyzed juice added (+H <sub>2</sub> O to make 10 cc.)	Color obtained upon adding iodine		
	Amount of NaSCN per 100 cc. of reaction-mixture		
	None	10 mg.	100 mg.
1.00	Blue	Blue	Blue
1.25	"	"	"
1.50	"	"	"
1.91	"	"	"
2.45	Bluish Yellow	Bluish Yellow	"
3.00	"	"	"
3.83	"	"	Bluish Yellow
4.80	"	"	"
6.00	"	"	"
7.50	"	"	"

constant at one cc. of one per cent Lintner's soluble starch and the variable was the amount of dialyzed juice which was added. Tables IV and V show that only with the highest amounts of NaSCN added, i.e., 96 mg. in Table IV and 100 mg. in Table V, was there any certain difference be-



tween the lots with and without thiocyanate, and this difference was one showing retardation by thiocyanate.

#### COMPARISON OF THE SACCHAROGENIC AND AMYLOLYTIC RESULTS

The experiments on the disappearance of the blue color with iodine corroborate the results of the experiments on the rate of formation of reducing sugars in indicating an unfavorable effect of thiocyanate upon the amylase activity; but the differences shown by added thiocyanate were much smaller by the iodine-color tests than by the method which measured the amounts of reducing sugars formed. Thus, amounts of thiocyanate which showed a definite retardation in the reducing sugars formed showed colors by the iodine test that were not distinguishable from those of the check lots containing no thiocyanate. This is due probably to the differences in the two methods and not to differences in the effect of the thiocyanate upon amylase activity. This question was studied by Evans (7) who pointed out the unsuitability of the iodine-color method for quantitative comparison and says (7, p. 221): "if in two cases there is even an apparently negligible difference in the iodine reaction, yet the effect on the substrate is by no means negligible as is seen by comparing the cupric reducing powers in the two cases"; and he states further that: "with weaker enzymes the final state of equilibrium is attained with extraordinary slowness, as though the small amount of enzyme encountered an almost insuperable resistance toward the end of the reaction." The results of the present experiments are in accord with the view of Evans; the iodine-color method is not suitable for distinguishing differences in amylase activity of such a weakly-acting liquid as potato juice. As to the question of a resistance encountered toward the end of the reaction, it seems more likely that this is related to the low concentration of starch that is present in the reaction-mixture as the end-point is approached.

#### SUMMARY

1. Since previous experiments had shown the effectiveness of solutions of thiocyanates in hastening the germination of freshly-harvested potato tubers, and since the successful treatments also caused a reduction in the starch content of the tubers, experiments were undertaken to determine whether this result could be accounted for by a direct effect of the thiocyanate in hastening amylase activity.

2. Experiments were carried out with both press-juice and juice that had been dialyzed in collodion bags. Two methods of measuring amylase activity were used: (a) by the increase in reducing sugars in the reaction-mixtures (saccharogenic action); (b) by the rate at which the starch was broken down into substances not giving a blue color with iodine (amylolytic action).

3. With press-juice the saccharogenic activity was decreased by both NaSCN and KSCN when the amounts added were in excess of about 10 mg. per 100 cc. of reaction-mixture. Smaller amounts of thiocyanates had no effect. The amylolytic activity was influenced to a much less extent, it requiring about 100 mg. to cause a definite retardation. It is suggested that the amylolytic method is unsuitable for detecting small differences in the amylase activity of potato juice.

4. The results with juice which had been dialyzed in collodion bags before the addition of the thiocyanate were similar to those with the press-juice.

5. Stimulative effects upon potato amylase by thiocyanates were not observed. Within the limits of the amounts of thiocyanates absorbed by potato tissue in the chemical treatments, (not more than 10 mg. per 100 cc. of juice) no effects were noted. Consequently, it is believed that neither the sprouting response nor the observed breakdown of starch of potatoes treated with thiocyanates is related to any direct effect which the chemical itself exerts upon the amylase of the potato.

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## THE EFFECT OF THIOCYANATES UPON AMYLASE ACTIVITY. II. SALIVARY AMYLASE<sup>1</sup>

LAWRENCE P. MILLER

In view of the failure to find an activating effect of thiocyanate upon potato amylase, as shown in the preceding paper (2), it seemed desirable to present the evidence that had been obtained on the question whether thiocyanate has any favorable effect upon salivary amylase.

Saliva contains small quantities of thiocyanate, but the authors who have reported upon the effect which it may exert upon the amylase of saliva have expressed wide differences of opinion. Thus, Dryere (3) found no effect of thiocyanate upon the rate of hydrolysis of starch by salivary amylase as measured by the length of the chromic period. Biltorf and Falkenhäusen (1) found that KSCN activates dialyzed saliva to some extent as do many other neutral salts, but that only in the presence of chlorides does KSCN exert a pronounced activating action; this action the authors regard as a specific function of thiocyanate. Rockwood (8) reported a slight increase in the rate of formation of reducing sugars in the presence of 0.01 molar  $\text{NH}_4\text{SCN}$ . Johnson and Wormall (6) found that even small amounts of KSCN hasten the breakdown of starch. They report, however, that this stimulative effect occurs only in the early stages of hydrolysis, i.e., when the course of digestion is followed by the color changes shown by the test with iodine (blue, purple, wine, red, yellow). They found no difference in the rate of formation of reducing sugars in the presence and in the absence of KSCN.

The results of the present experiments indicate that the conclusions described above should be modified in the following respects: at the pH of saliva it is only in the absence of chloride that small amounts of thiocyanate activate the amylase; the amounts of thiocyanate which hasten the starch changes as shown by color tests with iodine also increase the rate of formation of reducing sugar; the question whether thiocyanate activates saliva, depends upon the pH and upon the concentration of thiocyanate; by suitably varying these two factors, increases, decreases, or no effect can be found.

### METHODS

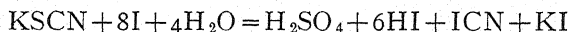
Three methods were used in this study of the effect of KSCN upon amylase activity: (a) determination of the rate at which reducing sugars were formed; (b) determination of the rate at which starch, which gives a blue color with iodine, was broken down into simpler polysaccharides,

<sup>1</sup> Herman Frasch Foundation for Research in Agricultural Chemistry, Paper No. 20.

which give violet, wine-colored, red, or yellow colors with iodine; (c) determination of the rate at which the viscosity of a mixture of starch, amylase, buffer, etc. was diminished.

*Change in reducing sugars.* The reaction mixture was prepared as follows: To 20 cc. 2 per cent soluble starch were added 20 cc. M/15 phosphate buffer of required pH, 5 cc. water or thiocyanate solution, and 2 cc. of saliva which had been diluted usually 1 to 40. The saliva had previously been dialyzed for about 18 hours against distilled water and was free of chlorides. A series of such mixtures was prepared in centrifuge tubes of about 80 cc. capacity. The reaction was carried out at 27° C. in a constant temperature water bath. After the hydrolysis had progressed to the stage at which considerable reducing sugar had been formed the reaction was stopped by the addition of Fehling's solution. The tubes were then placed in an 80° C. bath for 30 minutes, centrifuged, the cuprous oxide boiled with water, again centrifuged, and the copper determined by dissolving it in ferric ammonium sulphate and sulphuric acid and titration with potassium permanganate. It is important that all the thiocyanate be washed out since it reacts with permanganate. When large amounts are present it is necessary to boil the  $\text{Cu}_2\text{O}$  with water several times. The amount of reducing sugar present is expressed in terms of N/20  $\text{KMnO}_4$ .

*Color change with iodine.* The iodine tests were made upon samples taken from the same mixtures that were used in the reducing sugar experiments. At intervals, aliquots of 5 cc. were pipetted into test tubes containing 1 cc. of 10 per cent trichloroacetic acid and the color after the addition of iodine was observed. In making this test it is important that the liquid to be tested is acid in reaction and that the proper amount of iodine is added. If only a drop of dilute iodine is added the resulting color may not give a true measure of the stage of starch hydrolysis for the reason that the thiocyanate itself absorbs iodine. The reaction



forms the basis of an iodometric method for the estimation of thiocyanates proposed by Rupp and Schied (9). This reaction takes place most readily in an alkaline medium, but only slowly at neutrality. In the presence of the buffers which were used, however, iodine was continually being absorbed by the solutions containing KSCN. Thus, with a series of solutions containing starch or mixtures of starch and dextrans and increasing amounts of KSCN, it was possible to secure a series of colors, which might indicate an activating effect due to KSCN, but which could be obtained in the absence of enzyme as well, and was in fact due to the varying amounts of excess iodine present in the solutions. The amount of excess iodine was found to be especially important in mixtures of starch and dextrans; when a small amount of iodine was present, the blue color of starch resulted;

when more iodine was added, the red color of dextrin predominated. Also in the presence of KSCN the first shade of color observed with iodine in the presence of pure starch was always purple (violet), and this purple could not be distinguished from the purple which results from certain mixtures of starch and dextrans.

If the iodine test is made in an acid solution, the interference due to KSCN is not significant unless large amounts of KSCN are present. It was found that when 5 cc. of substrate solution were pipetted into 1 cc. of 10 per cent trichloroacetic acid before the iodine was added, the KSCN did not interfere with the color obtained unless present in concentrations above 0.5 per cent or 0.05 M. At concentrations below this, the color obtained with iodine at the beginning of the reaction before an appreciable amount of starch was broken down, was a blue apparently the same as that obtained where no KSCN was present. When larger amounts of KSCN were present and the initial color of the mixture was purple or reddish, no attempt was made to draw any conclusions from color data.

*Viscosity changes.* For the study by viscosity changes the solutions were made up as follows: To 50 cc. of 3 per cent starch were added 10 cc. of phosphate buffer, 10 cc. water or thiocyanate solution, and 10 cc. saliva (usually diluted 1 to 80). After mixing, 6.7 cc. were introduced into an Ostwald viscosity pipette and readings were taken at frequent intervals. The results are shown graphically by plotting the time in minutes after the reaction started against the number of seconds necessary for the bulb of the viscosity tube to empty. The temperature was kept constant at 25° C. by means of a water bath controlled by a Freas thermostat.

## EXPERIMENTAL RESULTS

### RESULTS OF MEASUREMENTS OF THE AMOUNT OF REDUCING SUGARS FORMED

#### *Measurements at the pH of Saliva*

*In the absence of NaCl.* The effect of KSCN upon the amylase activity of saliva was first tested at pH 6.9, which is within the pH range of normal saliva (5), and in the absence of NaCl. The results are shown in Figure 1 in which the concentrations of added KSCN are given as abscissas and the amylase activities expressed as cubic centimeters of  $\text{KMnO}_4$  solution resulting from reducing sugars formed as ordinates. It is seen that even small amounts of KSCN increased the amylase activity; that when the KSCN was present in an amount equal to 0.01 M the activity approximately doubled; and that when the amount was increased to 0.08 M the reducing sugar formed increased about five-fold. The amounts of KSCN ordinarily present in saliva are given by Reissner (7), from whose data it is found that the thiocyanate concentration is about 0.004 to 0.035 per cent. An examination of the curve in Figure 1 shows that within the range of con-

centration normally present in saliva, it is possible that the thiocyanate may hasten amylase activity, provided sodium chloride is absent.

*In the presence of NaCl.* It does not follow, however, that if KSCN activates dialyzed saliva, it has a similar function in normal saliva. It is well known that the animal amylases are inactive in the absence of salts. According to Ernström (4) the concentration of NaCl optimum for the action of salivary amylase is about 0.1 per cent, which is within the range

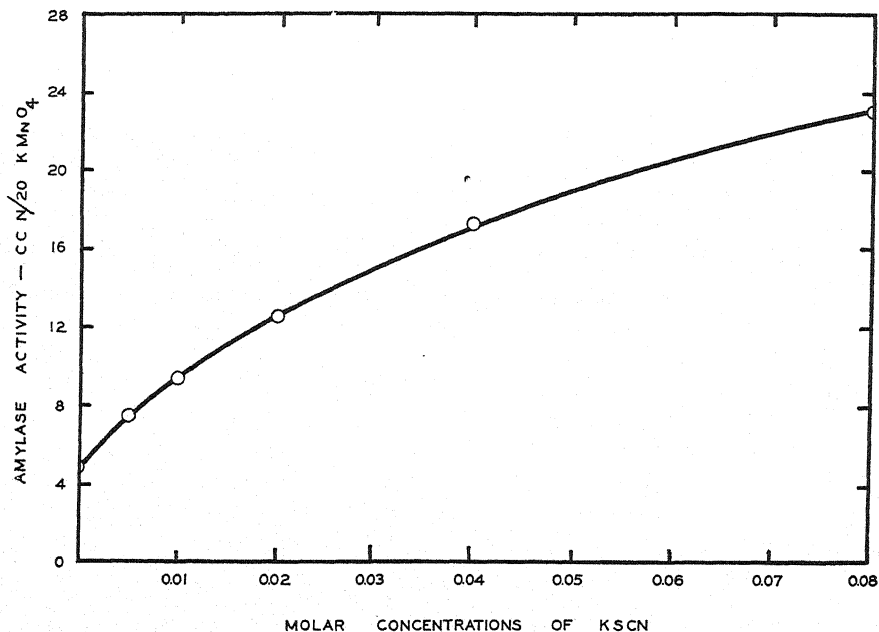


FIGURE 1. The effect of various concentrations of KSCN on the amylase activity of dialyzed saliva at pH 6.9 as measured by the reducing sugars formed.

present in normal saliva, that is, between 0.05 and 0.2 per cent. When concentrations of KSCN, which had a definite activating influence in the absence of NaCl, were added to substrate mixtures containing sufficient quantities of NaCl for optimum activation, the KSCN was found to have no effect. In Figure 2 are shown the results of two such tests with two different lots of saliva in the presence of 0.11 and 0.27 grams NaCl per 100 cc. and at a pH of 6.6 and 6.8 respectively. It is seen that when these amounts of NaCl are present KSCN could be added in amounts up to 0.06 M without having any effect upon the amylase activity of the saliva.

#### *Measurements at Various pH Values*

In order to study more fully the effect of KSCN upon dialyzed saliva in the absence of NaCl, a series of tests was made in which the pH of the

solution was varied from about 5.4 to about 7.5, and in which the concentration of KSCN varied from 0.001 M to 0.5 M. The results are shown in Figure 3. It is seen that the optimum pH for the amylase activity was influenced by the amount of KSCN that was present, and that as the amount of KSCN was increased the optimum pH was shifted toward the alkaline side. Thus, in the absence of KSCN (curve marked H<sub>2</sub>O in Fig. 3) the optimum pH was at a point more acid than pH 5.7, with 0.01 M KSCN it was at 6.0, with 0.075 M it was at 6.5, and with 0.5 M at 6.8. Figure 3

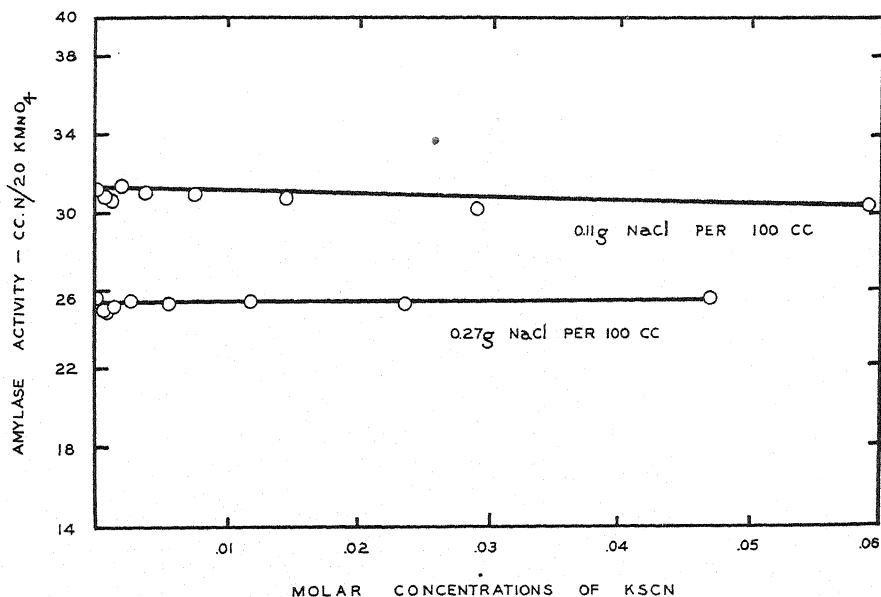


FIGURE 2. The effect of KSCN on the amylase activity of dialyzed saliva in the presence of 0.11 and 0.27 grams NaCl per 100 cc. and at pH 6.6 and 6.8 respectively.

shows further that the question whether KSCN hastens amylase activity can be given different answers, depending upon the concentration of KSCN and upon the pH. Thus, at high pH values KSCN increased the activity; at low pH values it depressed, at least at concentrations between about 0.1 and 0.5 M; at intermediate pH values no effect at all may be observable, as is shown, for example, in the curves for 0.5 M, 0.001 M, and H<sub>2</sub>O at pH 6.0, and also the curves for 0.01 M and 0.5 M at pH 6.2. The range of pH values shown in Figure 3 is that obtainable with the phosphate buffer; consequently, studies at more acid pH values would have required the use of a different type of buffer. A more extensive survey of this factor was not within the needs of the present study.

It is interesting to note that salivary amylase is active in high concentrations of KSCN, the highest concentration at which hydrolysis still takes



place depending upon the pH, as would be expected from a consideration of the pH curves. In a determination, not shown in Figure 3, at pH 6.00 the amylase became inactive at a concentration of KSCN that was 0.62 M, while at pH 7.95 the amylase was still more active than the check in a concentration 2.7 M or 26 grams per 100 cc.

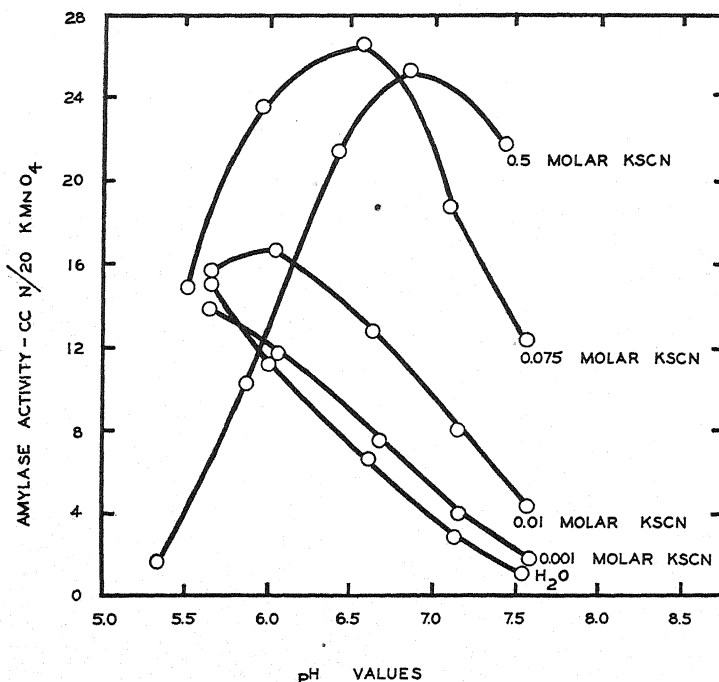


FIGURE 3. The effect of various concentrations of KSCN on the optimum pH for the amylase of dialyzed saliva.

#### RESULTS OF COLOR TESTS WITH IODINE

Johnson and Wormall (6) emphasized that in their experiments the stimulating effect of added KSCN was shown only by the color tests with iodine and not by an increase in the amount of reducing sugar formed. They believe that the KSCN effect, therefore, is only upon the early stages in the hydrolysis of the starch into simpler forms of polysaccharides.

In our experiments, as shown by the previous paragraphs, the KSCN under the proper conditions definitely resulted in an increase in the reducing sugars, and in all these experiments the course of the hydrolysis was followed also by color tests with iodine. In no case was any difference in color observed which was not also shown by the reducing sugar data.

## RESULTS OF MEASUREMENTS OF VISCOSITY CHANGES

The effects of KSCN upon the amylase activity of saliva, as shown by measurements of the change in viscosity, are shown in Figures 4, 5, and 6. The time in minutes from the start of the experiment is shown on the base line, and the time in seconds required for the flow of liquid through the viscometer is shown as ordinates. Consequently, in Figures 4, 5, and 6 the more rapidly the curves descend the more rapid is the decrease in viscosity, and, hence, the more active is the amylase in the solution. Thus, in Figure 4 are shown the readings at pH 6.9 in which the rate of change in

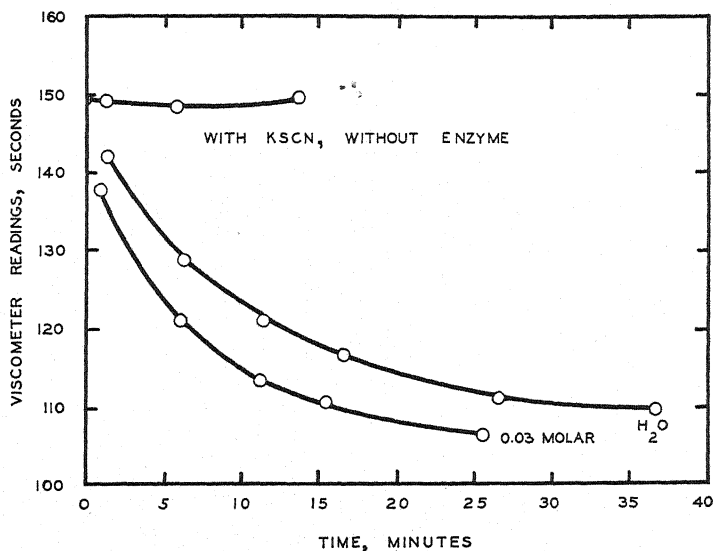


FIGURE 4. The effect of KSCN on the amylase activity of dialyzed saliva at pH 6.9 as measured by viscosity changes. In all the curves shown the original viscometer readings before any action on the starch equals that of the solution without enzyme.

viscosity of the check lot is shown in comparison with the lot containing KSCN at a concentration of 0.03 M; it is seen that the amylase activity as measured by the change in viscosity has been increased by the presence of the KSCN. However, in Figure 5 measurements at pH 5.8 show that the addition of KSCN to give concentrations of either 0.013 M or 0.64 M depressed the amylase activity. This is corroborative of the results given in a previous paragraph regarding the formation of reducing sugars, namely, that at the higher pH values KSCN hastens and at lower pH values it decreases the amylase activity of saliva.

If KSCN was especially active in hastening the first stages of starch hydrolysis, as believed by Johnson and Wormald (6), this should be brought out by the viscosity data since the drop in viscosity takes place at a very

early stage of the breakdown of starch before any color change can be noted and before any appreciable amount of reducing sugar is formed, as shown by Samec (10, p. 295). In none of our experiments could we find any effect of KSCN on amylase activity as measured by viscosity changes that could not also be demonstrated at a little later stage of the hydrolysis by reducing sugar determinations.

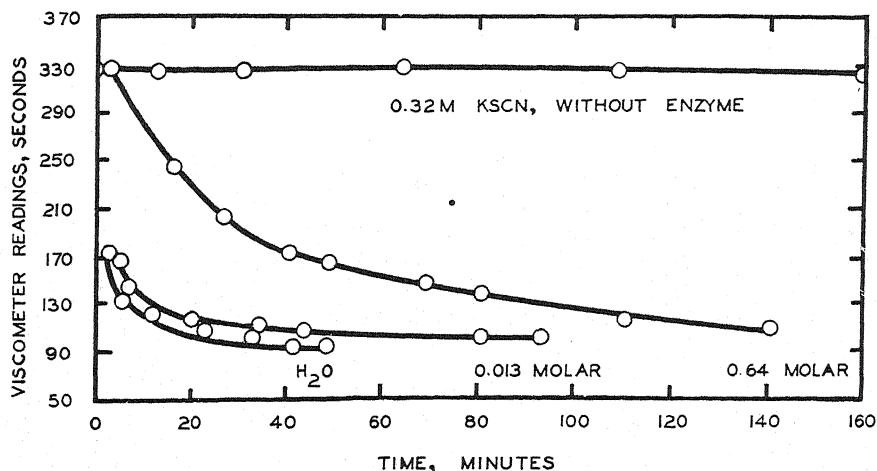


FIGURE 5. The effect of KSCN on the amylase activity of dialyzed saliva at pH 5.8 as measured by viscosity changes.

In order to determine the effect of KSCN upon the optimum pH for amylase activity as measured by viscosity changes, tests were made at different pH values in the presence and absence of thiocyanate. The results are shown in Figure 6, the several curves at the top showing the viscosity effect at different pH values in the absence of KSCN, and those at the bottom showing the viscosity when KSCN at a concentration of 0.64 M was present. In the absence of KSCN it is seen (top curves, Fig. 6) that the drop in viscosity values was more rapid with decreasing pH values, the most favorable pH for the amylase activity being that of the most acid value tested, i.e., pH 5.83. In the presence of 0.64 M KSCN, however, (bottom curves, Fig. 6) the most acid value, i.e., pH 5.54, was the least favorable, the optimum value being at pH 6.96. This shows again that the addition of KSCN to the solution shifts the optimum pH for salivary amylase toward the alkaline side as compared with the check lot containing no KSCN. The viscosity readings, therefore, gave results similar in all respects to those obtained by measurements on the rate of formation of reducing sugars.

It is interesting to note from the viscosity curves in Figures 4, 5, and the lower curves in Figure 6, that the difference in viscosity between the

check lots containing no KSCN and those in which KSCN was present was observable from the very first reading, or at least within a few minutes thereafter. This could not have been due to a direct effect of KSCN upon the viscosity of starch independently of any amylase effect, because the

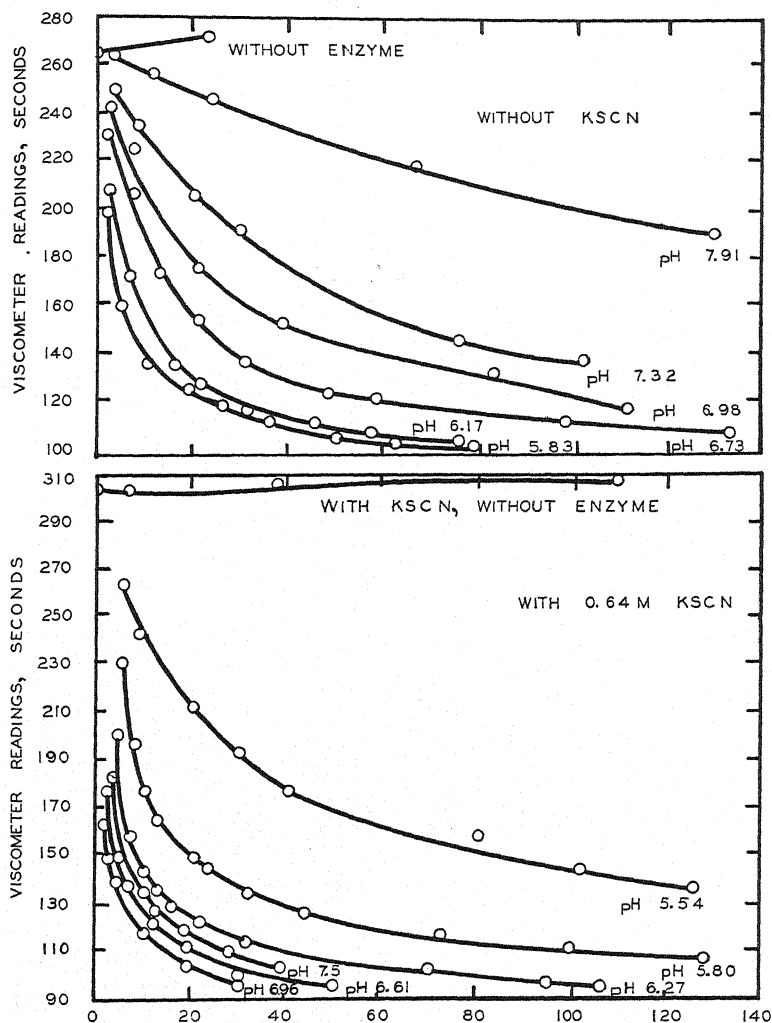


FIGURE 6. Curves showing the effect of KSCN in shifting the optimum pH as measured by changes in viscosity. Upper curves, without KSCN, lower curves, in the presence of 0.64 M KSCN.

curves marked "without enzyme" in Figures 4, 5, and 6 (lower curve only in Fig. 6) do not show this drop in viscosity although KSCN was present. Hence, the lowering of viscosity was produced by the thiocyanate affecting

the amylase reaction with starch and not by any direct effect of the thiocyanate upon the viscosity of starch.

#### SUMMARY

1. In the absence of NaCl, and at the approximate pH of saliva, the addition of KSCN to give concentrations from 0.005 M to 0.08 M caused increases in the amylase activity of saliva.

2. In the presence of NaCl in concentrations approximately that normally found in saliva and at pH 6.6, additions of KSCN to give concentrations up to 0.06 M produced no effect upon the amylase of saliva.

3. With increasing concentrations of KSCN the optimum pH for the hydrolysis of starch by dialyzed salivary amylase, in the presence of phosphate buffers, was shifted toward the alkaline side; this shift may be as much as a whole pH unit with high concentrations.

4. Potassium thiocyanate may depress, or stimulate, or have no effect on the amylase activity depending upon the pH at which the reaction takes place and upon the concentration of KSCN. At low pH values all concentrations of KSCN depress, at high pH values all concentrations stimulate, and at certain intermediate pH values KSCN may depress, or stimulate, or have no effect, depending upon the concentration.

5. The action of KSCN in affecting the amylase hydrolysis of starch was found to be essentially the same, whether measured by viscosity changes, color with iodine, or the formation of reducing sugars.

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# THE EFFECT OF POTASSIUM CYANIDE UPON THE AMYLASE ACTIVITY OF POTATO JUICE<sup>1</sup>

F. E. DENNY

## INTRODUCTION

In a preliminary note (2) increases in the amylase activity of the juice of potato tubers (*Solanum tuberosum* L.) after the addition of small amounts of KCN were described. The present paper reports the results of a more extensive series of experiments upon the same subject.

Attention was directed to this problem by the reports of Hanes and Barker (5, 6) that under suitable conditions cyanide increased the activity of malt amylase. This seemed important in connection with the experiments on the chemical treatment of dormant potato tubers, tests having shown (3) that the chemicals used, when added to potato juice, either decreased enzyme activity or had no measurable effect upon it; none exhibited any capacity to increase it.

Consequently tests were made of the effect of added KCN upon the amylase activity of potato juice. It was found, indeed, that small amounts of KCN when added to potato juice did increase markedly the amylase activity. It was noted, however, that if the juice was dialyzed before the KCN was added, little or no effect upon the amylase was found, the difference decreasing as the period of dialysis was increased.

Although these experiments with KCN were started for the purpose of obtaining information on the effect of chemical treatments on dormant potatoes, the results seemed to be of interest quite apart from these chemical treatments and to justify a description in a separate paper. The present paper, therefore, deals only with the effect of KCN upon the amylase activity of potato juice. The relation of these facts to the problem of chemical treatments of dormant potato tubers is to be described in a subsequent paper.

## METHODS

*Measuring amylase activity.* Amylase activity was measured by determining the gain in copper-reducing power of juice in contact with soluble starch as compared with a similar lot of juice to which water instead of soluble starch was added. The reducing sugar determinations were carried out by the Munson and Walker method (1, p. 78) and the cuprous oxide formed was titrated with a solution of potassium permanganate. The amylase activity was expressed as the number of cc. of  $\text{KMnO}_4$  solution representing the difference in the amounts of reducing sugars formed in the

<sup>1</sup> Herman Frasch Foundation for Research in Agricultural Chemistry, Paper No. 21.

presence and in the absence of soluble starch. The  $\text{KMnO}_4$  solution was standardized with dextrose and was of such concentration that 1 cc. of the  $\text{KMnO}_4$  solution was equivalent to 1.58 mg. of dextrose.

In order to determine whether the KCN had any effect upon the formation of reducing substances in the juice itself in the absence of starch, a special test was carried out in which the amounts of KCN used in these experiments were added to juice to which no starch was added. No gain in reducing power due to the presence of KCN was found; any observed effect of KCN, therefore, was upon the amylase-system and not upon other systems which could bring about an increase in the amount of copper-reducing substances in the juice.

The possibility that the KCN itself was having an effect upon the determinations of reducing sugars was taken into account. It was found, however, that the amounts of KCN used in these experiments did not change the permanganate titration values of potato juice in the sugar determinations. Furthermore, in the later experiments, at the end of each amylase test just before the sugar determinations were made, the amounts of KCN in all lots were equalized by adding to each lot an amount of KCN sufficient to equal that in the lot to which the largest amount of KCN had been added at the start of the experiment. In this way the amounts of KCN varied in the different lots during the progress of the experiment but were the same in all lots at the time the sugar determinations were made.

*Regulation of pH.* A solution of KCN is alkaline, and when more than about 10 mgs. of KCN per 100 cc. of a reaction-mixture (potato juice plus starch solution) is added the pH value is changed in an alkaline direction. In the early experiments this difficulty was overcome by adding to the KCN solution enough acid to bring it to the same pH as that to which the juice had been adjusted; in such cases the buffering power of the juice sufficed to maintain the constancy of the pH. In later experiments it was found that, if the KCN solution was neutralized to litmus paper by adding a solution of  $\text{H}_2\text{SO}_4$ , amounts of cyanide up to at least 100 mgs. per 100 cc. of reaction-mixture could be added without causing any change in the pH. In certain experiments, in which it was desired to study the effect of cyanide in the absence of buffer, this method was used, but in most of the experiments not only was the cyanide solution neutralized first but a phosphate buffer was added also. It was found that a total final phosphate concentration of about 0.05 M did not interfere with the amylase activity or with the reducing sugar determinations, and that it maintained the pH at a constant value. In all experiments in which tests were made at only one pH value, pH 6.2 was selected for the reason that the pH value of potato juice is usually found to be within the range 6.0 to 6.4.

*Experimental errors.* The error in the final amylase value when aliquots were taken from a given lot of juice was found to be about 4 per cent of the

average amylase value for that lot of juice. But the differences between lots of juice pressed on different days were much greater, the amylase values from the same lot of tubers ranging from about 4 cc. to 6 cc. of  $\text{KMnO}_4$  gain per cc. of the original potato juice used in preparing the reaction-mixture. It seems unlikely that this difference in values in different experiments is due to actual changes in amylase activity within the tuber from day to day, but is probably related to differences arising in preparing the sample of ground tissue and in pressing it. For example, a special experiment on the distribution of the amylase activity in different parts of the potato showed a value of 5.9 cc. for the juice from the outer layer of the tuber including the peel, 4.3 cc. for the juice from the layer between the core and the outer layer, and 2.9 cc. for the juice from the core; these values are cc. of  $\text{KMnO}_4$  per cc. of juice taken for the amylase test. The pressure applied in squeezing a sample of ground tissue also had an influence upon the amylase of the juice; thus, juice obtained by gentle hand-pressing gave a value of 5.1 cc., while that obtained by subjecting the residue to still further pressure in a screw press gave a value of 2.4 cc. In these experiments, however, uniformity in the activity of different pressings was not an important factor since the principal question related to the effect of the addition of KCN to any given lot of juice. In such cases care needed to be taken with each lot of juice only to keep all the conditions comparable except as to the presence and absence of KCN. In these experiments, therefore, differences between the amylase activities of juices of different pressings are not important, but only differences that were obtained from different aliquots of juice from the same pressing.

*Dialysis.* Since the results with dialyzed juice exerted an important influence upon the conclusions reached as a result of these experiments, the method of preparation of membranes and the procedure during dialysis are described with considerable detail. The collodion solution was prepared by mixing 5 grams of "Union Cotton Negative" in a mixture of 50 cc. of 95 per cent ethyl alcohol, 50 cc. of ethyl ether, and 5 cc. of glacial acetic acid. About 40 cc. of this collodion preparation were poured into a clean dry 100 cc. Pyrex test tube and were poured off again at once; the tube was held, mouth downward, at an angle of  $45^\circ$  from the perpendicular, and was rotated until the liquid detached itself slowly by drops from the rim; the tube while still inverted was warmed by rubbing with the hands until the inner surface, when touched with the finger, was found to be firm; water was added to the tube and was poured off; the collodion bag was detached from the inner surface of the tube; a cork was fitted into the opening, and the bag was securely tied with string, care being taken that no particles of saliva from the mouth or hands touched the bag at any time; 35 to 40 cc. of potato juice were poured into the bag through an opening in the cork, and the bag was suspended in water until only the



portion above the lower edge of the cork was above water; the tap water was continuously saturated with toluene before it passed around the colodion bags.

### EXPERIMENTAL RESULTS

#### EFFECT OF KCN UPON AMYLASE ACTIVITY OF POTATO JUICE

The effect of KCN upon the amylase activity of potato juice is shown in Tables I and II. For the experiment the results of which are shown in Table I no buffers were used, but the proper amount of dilute  $H_2SO_4$  was added to the KCN solution in order that when mixed with the potato juice the final pH would be that shown at the heads of columns 2 to 6 in Table I; the amounts of acid needed to furnish this adjustment had been determined in a preliminary test with the same juice; the juice lots to which no KCN was added were adjusted to the corresponding pH values by the addition of dilute acid or alkali; care was taken that the final volume of all lots after dilution was the same. Twenty cc. portions of these mixtures of juice, with and without KCN, were added to 20 cc. of 3 per cent soluble starch; the test tubes containing the reaction-mixtures were rotated for 64 hours on a turning-bar inside an oven whose temperature was regulated at  $35^\circ C$ . At the end of the experiment enough boiling 95 per cent alcohol was added to furnish a concentration of 70 per cent alcohol (by volume); after this was boiled and cooled it was made up to volume, filtered, and an aliquot taken; the alcohol was driven off by evaporation on a steam bath; the residue was made up to volume with water; and aliquots were taken for the reducing sugar determination. In Table I the amylase

TABLE I  
EFFECT OF KCN UPON THE AMYLASE ACTIVITY OF POTATO JUICE

KCN added per 100 cc. of reaction- mixture, mg.	Amylase activity, expressed as cc. of $KMnO_4$ solution; at the following pH values				
	pH 6.00	pH 6.30	pH 6.50	pH 6.75	pH 7.00
None	6.8	12.3	15.8	20.1	19.9
10	14.2	21.8	26.9	29.6	26.4
20	16.5	26.0	29.2	30.4	30.7
30	16.5	26.9	30.4	32.3	32.6
40	18.2	30.3	32.7	32.9	31.5
50	22.0	31.7	32.1	34.3	32.7

activities of the various lots are given in cc. of  $KMnO_4$  solution per 2.67 cc. of the original juice, these values being obtained by deducting the values of the blank lots not containing starch from those of the corresponding lots which contained starch. Column 1 in Table I shows the number of milligrams of KCN per 100 cc. of reaction-mixture added in each case. The results show that the addition of 10 to 50 mgs. of KCN per 100 cc. increased

the amylase activity at all pH values in the test; the differences were somewhat greater at pH values of about 6.3 to 6.5 than at either 6.0 or 7.0; at pH 7.0 the effect of differences in the amount of added KCN was small, the values being about the same whether 20, 30, 40, or 50 mgs. of KCN were added.

Additional results regarding the effect of KCN are shown in Table II. This experiment differed from that described previously mainly in the fact that a phosphate buffer was added to give further assurance that the pH values were maintained constantly at the desired value. In this experiment the reaction-mixture consisted of 25 cc. of juice after adjustment to the pH values shown in Table II, plus 10 cc. of H<sub>2</sub>O or of previously neutralized KCN solution containing amounts of KCN sufficient to give the values shown in column 1 in Table II, plus 25 cc. of 0.2 M phosphate buf-

TABLE II  
EFFECT OF KCN UPON THE AMYLASE ACTIVITY OF POTATO JUICE

KCN added per 100 cc. of reaction- mixture, mg.	Amylase activity, expressed as cc. of KMnO <sub>4</sub> solution; at the following pH values		
	pH 6.22	pH 6.68	pH 7.15
None	10.9	21.3	9.4
10	22.8	31.2	27.6
30	27.2	31.2	26.0
90	25.9	29.1	19.5
270	23.8	21.9	14.8

fer at the stated pH values, plus 30 cc. of 3 per cent soluble starch. The reaction tubes were rotated in a constant temperature oven for 44 hours at the end of which time the sugar determinations were carried out as described in a preceding paragraph. The results for three different pH values are shown in Table II, the values given being for an aliquot representing 2.0 cc. of the original juice before dilution. It is seen (Table II) that the added amounts of KCN increased the amylase activity in all cases except possibly for the 270 mg.-lot at pH 6.68. The most favorable amount was about 30 mgs. per 100 cc. of reaction-mixture, although at pH 7.15 possibly the smaller amount was more effective.

*Heavy Metals and Oxidase as Factors in the Cyanide Effect upon  
Potato Amylase*

*Heavy metals.* The effect of added KCN upon enzyme activity has been interpreted by certain authors as being due to a reaction between the KCN and ions of the heavy metals that are present. If *increases* were observed after adding KCN this was thought to be due to the action of the cyanide-ion in forming complexes with those heavy metals which exert an inhibiting effect when in the free condition (Krebs, 8). If *decreases* were found this

was regarded as evidence that the heavy metal atom was an essential constituent of the enzyme, and that the cyanide-ion by reacting with it retarded the enzyme action (Haldane, 4, p. 157). In the present experiments since the potato tissue had been in contact with a metal knife-blade in removing the peel, and with a metal food grinder in preparing the press-juice it was thought desirable to test the effect with juice that had been pressed without contact with metal. This was done by pounding the potato tubers in a porcelain mortar with a pestle and squeezing the juice through cheesecloth. The results are shown in Table III in which in columns 3 and

TABLE III  
EFFECT OF KCN UPON AMYLASE OF POTATO JUICE  
PRESSED WITHOUT CONTACT WITH METAL

Variety of potato	Buffer used	Amylase activity, expressed as cc. of $\text{KMnO}_4$	
		KCN not added	KCN added
Early Ohio	Phosphate	12.4	19.8
" "	None	12.4	20.8
" "	"	11.8	15.4
" "	Phosphate	21.5	42.5
Irish Cobbler	"	21.7	39.6
Bliss Triumph	"	16.0	31.1

4 are shown the amylase activities in the presence and in the absence of KCN; the values given are cc. of  $\text{KMnO}_4$  solution per 4 cc. of the original juice, these values representing the gain in reducing sugar as a result of contact with soluble starch; all experiments were carried out at pH 6.2 and the KCN lots received an amount of KCN equal to 30 mgs. per 100 cc. of reaction-mixture; in the experiments in which no buffer was used the juice was first adjusted to pH 6.2 and the KCN solution was neutralized to litmus paper before these two were added, under which conditions no change in pH occurs.

It is clear from Table III that the addition of KCN to these juices that were obtained without contact with metal increased markedly the amylase activity. These experiments show that, if the cyanide effect is related to reaction with heavy metal ions, these ions must be such as occur naturally in the press-juice of potatoes.

*Oxidase.* Woods (9) in his study of the mosaic disease of tobacco found that oxidizing enzymes interfered with amylase activity. This seemed important in connection with these experiments since, if KCN interfered with an oxidase which retarded amylase, the addition of KCN would increase the amylase values, not by any direct effect upon amylase, but indirectly by an effect upon oxidase. If this were true, then any substance which retarded oxidase should indicate an apparent increase in amylase.

Kastle and Loevenhart (7) showed that small amounts of sodium thio-sulphate on the order of 40 mgs. per 100 cc. of reaction-mixture destroyed the oxidase of potato. In the present experiments the effect of sodium thio-sulphate was tested and it was found that amounts of  $\text{Na}_2\text{S}_2\text{O}_3 \cdot 5\text{H}_2\text{O}$  varying from 10 to 160 mgs. per 100 cc. of reaction-mixture could be added without producing any effect upon the amylase activity. In order to obtain this result, the favorable effect of sodium thiosulphate in destroying the oxidase (and thus increasing amylase activity) must be nullified by an equally unfavorable effect directly upon the amylase itself.

These tests, although not successful in ascribing the cyanide effect to its influence upon oxidase activity, are not sufficient to exclude oxidase as a factor. Further experiments on this phase are needed, and the dialysis results that are described in later paragraphs indicate that by comparing the oxidase action during progressive dialysis with the cyanide effect upon amylase at each dialysis period, the importance of the oxidase factor could be studied effectively.

EFFECT OF KCN UPON AMYLASE ACTIVITY OF DIALYZED  
POTATO JUICE

*Dialysis over long periods.* The effect of KCN upon the amylase activity of potato juice after dialysis for 16 hours in running tap water and for 3 to 6 hours in running distilled water are shown in Table IV. It is seen that

TABLE IV  
EFFECT OF KCN UPON THE AMYLASE ACTIVITY OF  
DIALYZED POTATO JUICE

Experiment 1		Experiment 2			
KCN added per 100 cc. of reaction- mixture, mg.	Amylase activity, cc. of $\text{KMnO}_4$	KCN added per 100 cc. of reaction- mixture, mg.	Amylase activity, expressed as cc. of $\text{KMnO}_4$		
	Early Ohio		Bliss Triumph	Irish Cobbler	Early Ohio
None	18.9	None	21.8	21.1	20.6
5	19.5	3	20.0	20.5	23.7
15	19.2	9	23.2	22.6	23.4
45	18.4	27	23.4	21.9	24.0
135	22.6				

the increases that were observed to occur after adding KCN to press-juice were not found to occur when similar amounts of KCN were added to dialyzed juice. The differences in amylase values shown in Table IV are so close to the range of experimental error that it cannot be concluded that any differences at all are shown. This result suggested that the effect of KCN was not merely upon the amylase itself, but also upon some dialyz-

able substance or substances of the juice. In order that the cyanide effect could become evident, it seemed that there must be present not only the amylase and the cyanide, but also another fraction of the juice which takes part in the reaction and which can diffuse through collodion.

*Dialysis at intervals.* Since the activating effect of KCN was present before and absent after dialysis, experiments were undertaken to determine at what period during dialysis the loss in the favorable effect of KCN would become evident. The results are shown in Tables V and VI. In Table V the results are shown for intervals of dialysis up to 13 hours. The

TABLE V  
RELATION OF DURATION OF DIALYSIS TO THE KCN  
EFFECT UPON POTATO AMYLASE

Duration of dialysis, hours	Amylase activity, expressed as cc. of KMnO <sub>4</sub>		cc. KMnO <sub>4</sub> gain due to KCN
	KCN not added	KCN added	
0	15.9	28.2	12.3
1.5	17.2	31.3	14.1
4	18.9	29.4	10.5
7	22.7	29.1	6.4
10	19.1	24.1	5.0
13	21.0	24.3	3.3

tissue was ground and juices were pressed at such intervals that the periods of dialysis all ended at the same time; then the amylase tests were started simultaneously, and the rotation of the reaction-mixtures of enzyme and starch, with and without KCN, was continued for 48 hours. The amount of KCN added per 100 cc. of reaction-mixture was 30 mgs., and the test was carried out at pH 6.2. The amylase values in Table V are given per 4.0 cc. of the original juice. It is seen in column 4, Table V, that the favorable effect of KCN was maintained fairly well up to about 4 hours of dialysis after which the gain due to KCN became less pronounced. The differences between the check lots and the lots to which KCN was added decreased until at the 13th hour the gain was small.

Although the data in Table V show that the differences between the amylase activities of the lots with and without KCN decreased as the period of dialysis increased, they do not show the trend of the amylase activities in the two cases. This is due probably to the fact that a new lot of juice was prepared at each period at which a dialysis was started, and the variation in the amylase activities of juice from different pressings was too great to show the trend of amylase activity during the progress of dialysis. This point was tested in another experiment in which the different periods of dialysis were started simultaneously, aliquots being taken from the same pressing of juice; samples were then removed at intervals of 4, 8,

12, and 24 hours after the start of dialysis; the reaction-mixtures of enzyme and starch, with and without KCN, were prepared as soon as the dialysis of each lot was completed, and the period of rotation of the reaction-mixtures was exactly 48 hours from the end of dialysis. The results are shown in Table VI in which the values given represent the amylase ac-

TABLE VI  
RELATION OF DURATION OF DIALYSIS TO THE KCN  
EFFECT UPON POTATO AMYLASE

Duration of dialysis, hours	Amylase activity, expressed as cc. of $\text{KMnO}_4$		cc. $\text{KMnO}_4$ gain due to KCN
	KCN not added	KCN added	
0	21.9	34.7	12.8
4	21.1	27.6	6.5
8	21.2	27.1	5.9
12	19.1	18.4	-0.7
24	16.2	17.4	1.2

tivities per 4.0 cc. of the original juice. Columns 2 and 3 show the amylase activities at the end of each dialysis interval and column 4 shows the differences between the lots with and without KCN. It is seen that the difference decreased as the period of dialysis increased, and furthermore, that this decrease in difference was due to the fact that the amylase activity of the KCN lot decreased faster than that of the check lot. The KCN lot started with a high value but this value decreased as the dialysis proceeded. The check lot, although it started with a value below that of the KCN lot, maintained its amylase activity at this level until after about 10 hours of dialysis; then its value fell. At the 12th hour of dialysis the values with and without KCN became approximately equal.

#### COMPARISON OF RATE OF DIALYSIS OF THE EFFECTIVE FRACTION IN THE CYANIDE-EFFECT WITH THAT OF REDUCING SUGAR

During the course of these and of previous experiments, it had been noted that the reducing sugar dialyzed rapidly through the collodion bag, and that, in fact, after about 4 hours 75 to 95 per cent of the reducing sugar had passed through. And yet after 4 hours dialysis the gain due to added KCN was still evident. This suggested that the effective constituent in the cyanide effect upon amylase dialyzed through collodion more slowly than reducing sugar. The experiment, the results of which are shown in Table VII, was carried out to test this point. The test was made at pH 6.2, and the amylase values in Table VII are given per 3.2 cc. of the original juice. Column 3 shows the number of cc. of  $\text{KMnO}_4$  corresponding to the amounts of reducing sugar present at the start and after dialysis intervals of 2 and

4 hours. Column 6 shows the gains in amylase activity due to the addition of 30 mgs. of KCN per 100 cc. of reaction-mixture. It is seen that, although more than 90 per cent of the reducing sugars had passed through the collodion bags within 4 hours, the amylase gains due to added KCN were at least 50 per cent of the value before dialysis. The fraction whose presence is necessary for the activating effect of KCN upon amylase diffused

TABLE VII  
COMPARISON OF GAIN IN AMYLASE ACTIVITY UPON ADDITION OF  
KCN WITH THE RATE OF DIALYSIS OF REDUCING SUGARS

Variety of potato	Duration of dialysis, hours	cc. $\text{KMnO}_4$ due to the reducing sugars inside the collodion bag	Amylase activity, expressed as cc. of $\text{KMnO}_4$		cc. $\text{KMnO}_4$ gain due to KCN
			KCN not added	KCN added	
Bliss Triumph	0	31.8	14.1	23.3	9.2
	2	9.4	13.6	21.8	8.2
	4	0.1	19.4	25.2	5.8
Irish Cobbler	0	27.3	17.1	31.8	14.7
	2	8.6	19.5	30.3	20.8
	4	1.4	21.4	29.0	7.6
Early Ohio	0	12.4	21.5	35.1	13.6
	2	2.7	16.8	26.5	10.7
	4	0.9	19.5	28.7	9.2

through collodion at a slower rate than that of reducing sugars. Although the data in Table VII do not indicate that a very sharp separation of the activating factor from reducing sugar could be made by a dialysis which was made variable as to the time of dialysis, it is possible that better results could be obtained by varying not the time of dialysis but the degree of permeability of the membranes used.

#### SUMMARY

1. The addition of small amounts of potassium cyanide to the juice of potato tubers (*Solanum tuberosum* L.) increased the amylase activity. At a pH value of about 6.2, thirty mgs. of KCN per 100 cc. of reaction-mixture increased the activity by 50 to 100 per cent. At pH values approaching neutrality the KCN effect was less pronounced and the differences between different amounts of KCN were smaller.

2. The addition of KCN to potato amylase prepared by dialyzing potato juice in collodion bags for 24 hours, however, had slight or negligible effects upon the amylase activity.

3. When the juice was dialyzed for various time periods up to 24 hours before the addition of KCN, the gains in amylase activity due to the addition of KCN became progressively smaller.

4. In order that the activating effect of KCN can become evident, not only must the amylase and the KCN be present, but also a portion of the juice which will pass through collodion in dialysis.

5. Evidence was obtained indicating that the fraction of the potato juice whose presence is necessary for the activating effect of KCN upon amylase diffuses through collodion at a rate that is slower than that for reducing sugars.

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# THE INFLUENCE OF SULPHUR COMPOUNDS IN BREAKING THE DORMANCY OF POTATO TUBERS<sup>1</sup>

## PRELIMINARY REPORT

LAWRENCE P. MILLER

It has been found by Denny (1) that the rest period of dormant tubers of the potato (*Solanum tuberosum* L.) can be broken by treatment with suitable concentrations of ethylene chlorhydrin ( $\text{CH}_2\text{ClCH}_2\text{OH}$ ), sodium thiocyanate ( $\text{NaSCN}$ ), and thiourea ( $\text{NH}_2\text{CSNH}_2$ ). As can be seen from the formulas, these substances are quite unlike chemically. They are also very dissimilar in their physical properties. But with the additional information that carbon bisulphide ( $\text{CS}_2$ ) (1, 4) also has some effect in breaking dormancy, it is noted that these effective compounds are sulphur compounds with the exception of ethylene chlorhydrin. A study of the expressed juices of potatoes treated with ethylene chlorhydrin has shown that such treatment induces the formation of reducing substances, as measured, for example, by titration with iodine in acid solution (2). Titration with iodine in acid solution has been recommended by Tunncliffe (6) as a measure of glutathione. Guthrie (5) has reported that juices from chlorhydrin-treated potatoes have an increased capacity to form  $\text{H}_2\text{S}$  from sulphur. This reaction is characteristic of sulfhydryl compounds such as glutathione and cysteine. Of the four effective compounds under consideration then, three are sulphur compounds and the other one has a marked effect on the sulphur metabolism of the potato.

Tests with additional sulphur compounds are reported in this paper. It was desired to try sulphur compounds with various structural relationships in order to obtain some idea of the relative importance of certain groups in these compounds. This work is now in progress. Work so far completed has shown many sulphur compounds to be unusually effective.

In trying a new chemical if it was soluble in water a one-hour soak treatment was used; if insoluble, vapor treatments of 24 hours duration were used. The tests were made on various lots of Irish Cobbler, Bliss Triumph, and Early Ohio varieties of potatoes.

Certain of the compounds were prepared in the laboratory according to published methods. Whenever possible the desired compounds were obtained from the Organic Division of the Eastman Kodak Company.

## RESULTS

One of the sulphur compounds found to be very effective was ammonium dithiocarbamate ( $\text{NH}_2\text{CSSNH}_4$ ), which is related to thiourea since it

<sup>1</sup> Herman Frasch Foundation for Research in Agricultural Chemistry, Paper No. 22.

is the ammonium salt of dithiocarbamic acid ( $\text{NH}_2\text{CSSH}$ ), while thiourea is the amide of thiocarbamic acid ( $\text{NH}_2\text{CSOH}$ ). It is readily prepared from ammonia and carbon bisulphide (3, p. 201). If the reaction is carried out in a mixture of alcohol and ether, crystals of the salt soon separate out. The recrystallized salt did not differ in activity from the crude product thus obtained. This substance was tested on different varieties of dormant potatoes during the season of 1930 and was found to produce a prompt and uniform growth of buds. Figure 1 illustrates a test with Bliss Triumph po-

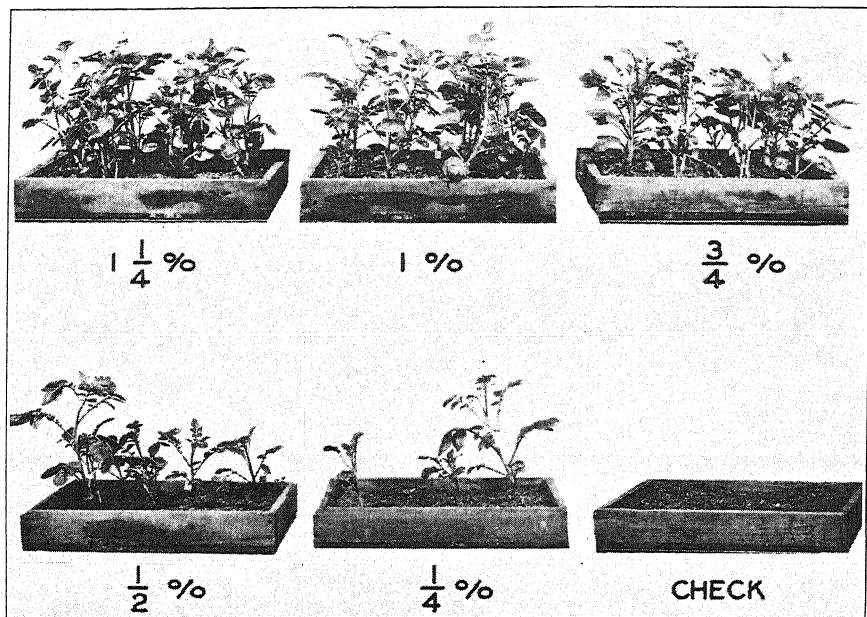


FIGURE 1. Effect of various concentrations of ammonium dithiocarbamate in breaking the dormancy of Bliss Triumph potatoes.

tatoes. It is seen that there is a rather wide range of effective concentrations, one and one-quarter, one, and three-quarters per cents giving very good results, while one-half and one-quarter per cent resulted in growth intermediate between the higher concentrations and the check in which no sprouts had appeared above ground when this photograph was taken, 42 days after treatment.

Other effective compounds are thiosemicarbazide ( $\text{NH}_2\text{CSNHNH}_2$ ), thioglycolic acid ( $\text{CH}_2\text{SHCOOH}$ ), thioacetic acid ( $\text{CH}_3\text{COSH}$ ), hydrogen sulphide ( $\text{HSH}$ ), methyl disulphide ( $\text{CH}_3\text{SSCH}_3$ ), ethyl mercaptan ( $\text{C}_2\text{H}_5\text{SH}$ ), thioglycol ( $\text{CH}_2\text{SHCH}_2\text{OH}$ ), and various derivatives of dithiocarbamic acid ( $\text{NH}_2\text{CSSH}$ ).

In Figure 2 are illustrated results with three of these chemicals. The upper row illustrates results with five-sixteenths cc. per liter of thioglycollic acid. In this series treatment with five-sixths cc. and five-eighths cc. per liter also gave good results. The middle row shows the effects of treatment with hydrogen sulphide gas, the concentrations of which were con-

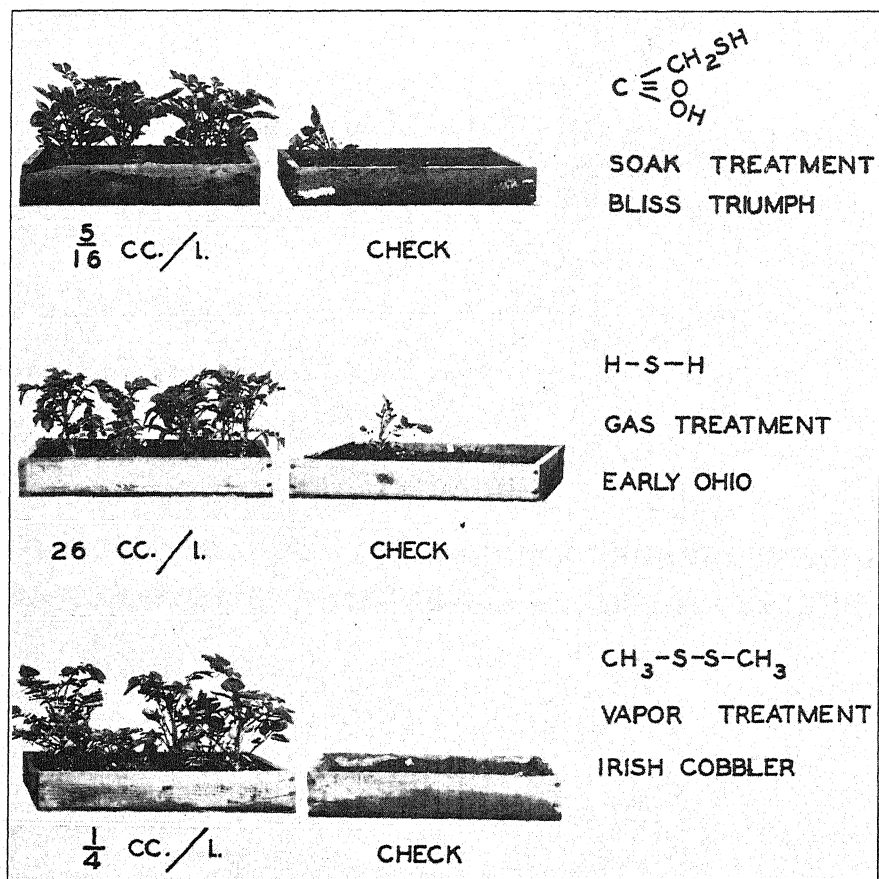


FIGURE 2. Effect of various sulphur compounds on dormant potatoes.

trolled by weighing out the proper amount of  $\text{Na}_2\text{S} \cdot 9\text{H}_2\text{O}$  and liberating the hydrogen sulphide with an excess of sulphuric acid. The effective range for hydrogen sulphide is quite narrow. In the particular series illustrated 26 cc. and 29 cc. per liter gave good results, while 36 cc. per liter was a toxic concentration and 20 cc. per liter was no better than the check. With methyl disulphide shown in the lower row, one-quarter cc. and one-six-

teenth cc. per liter were very effective, while treatments with one-half cc. and one cc. per liter showed some evidence of injury.

Details of these experiments as well as of further work now being done will be published later.

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## THE MOVEMENT OF GASES INTO AND THROUGH PLANTS

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Illuminating gas is known to have specific effects upon plants. Ethylene, which is the effective ingredient of illuminating gas, causes epinasty of tomato leaves when the concentration is as low as one part of the gas to 5,000,000 parts of air. In tomato and many other plants severe injury may result if the concentration of the gas is sufficiently high and the treatment lasts for several days. Plant species are not all equally sensitive to ethylene. For example, ferns exposed to 90 per cent ethylene for two days showed no injury, whereas tomatoes responded to traces of the gas. This variation has called forth considerable speculation as to how the ethylene gets into the tissues and whether or not the resistant organs are impervious to this gas. Detailed experiments to determine the exact effect of various gases on plants have been under way at the Boyce Thompson Institute for several years. One phase of this work was concerned with a comparison of the anatomy of normal and treated tomato leaves to find whether the downward movement was due to multiplication and elongation or simply elongation of cells. The size and arrangement of cells in normal petioles of different plants vary greatly and for that reason it was desirable to compare the anatomy of petioles adjacent on a stem. It was thought that one leaf could be gassed for 24 hours to cause a response and then it could be removed and compared with adjacent leaves. This procedure was attempted with the result that when one leaf was treated with illuminating gas all of the leaves on the plant showed the ethylene response. Apparently the gas entered the treated leaf and then spread throughout the plant. To make certain that the plant responded to gas diffusing from this one source instead of being affected by contaminated air of the laboratory, other similar experiments were performed in the greenhouse where plants grew normally. The effect was the same as in the laboratory. The results of several methods used to show that ethylene moves readily through seven different species of plants are reported in this paper.

### PROCEDURE AND RESULTS

As described in 1917 by Doubt (3), and more recently by Crocker (2), and Zimmerman, Crocker, and Hitchcock (4), tomato plants show a striking epinasty within a few hours after being exposed to illuminating gas. Under favorable conditions many of the leaves move downward until the petioles are nearly parallel to the stem of the plant. Young leaves are affected all along the upper surface, resulting in curling, but the older leaves are stimulated locally at the base of the petiole. The more rapid growth on the upper side of the petiole forces the whole leaf downward making it more

rigid than normal. In order to cause one leaf of the tomato (*Lycopersicon esculentum* Mill. var. Bonny Best) to make such a response without disturbing the rest of the plant a toy balloon was inflated with approximately 10 per cent illuminating gas and sealed over a petiole stripped of leaflets. After 18 hours, instead of only the treated leaf responding, all of the leaves were affected as if the whole plant had been incased with gas. Evidently the gas had passed from the balloon into the petiole and from there throughout the entire plant, causing all of the leaves to give the ethylene response.



FIGURE 1. Left: Tomato plant with a tube containing air fastened over a petiole, used as the control plant. Right: Tomato plant with a test tube containing illuminating gas sealed over a defoliated petiole. Note that the gas spread throughout the plant causing all parts to respond as if incased with gas.

There was some possibility, although not likely, that gas had leaked slowly from the balloon and had remained in the air about the plant long enough to cause the condition just described. Other experiments were then planned to make certain that no leakage occurred and also to determine whether the gas passed freely in both directions from the place where the treated petiole was attached to the stem.

Figure 1 shows the manner in which large glass tubes containing gas or

air were sealed over defoliated petioles at different positions on the stem. In order to prevent leakage sheet rubber was punctured, slipped over the petiole, and tied over the mouth of the glass tube. Control plants were set among the test plants so that if there was any leakage of gas into the air the controls would be equally affected with the treated plants. The treated plants gave the ethylene response while the check plants remained normal. One of these treated plants is shown in Figure 1. By sealing gas-filled tubes over petioles at various positions on the plant it was shown that the

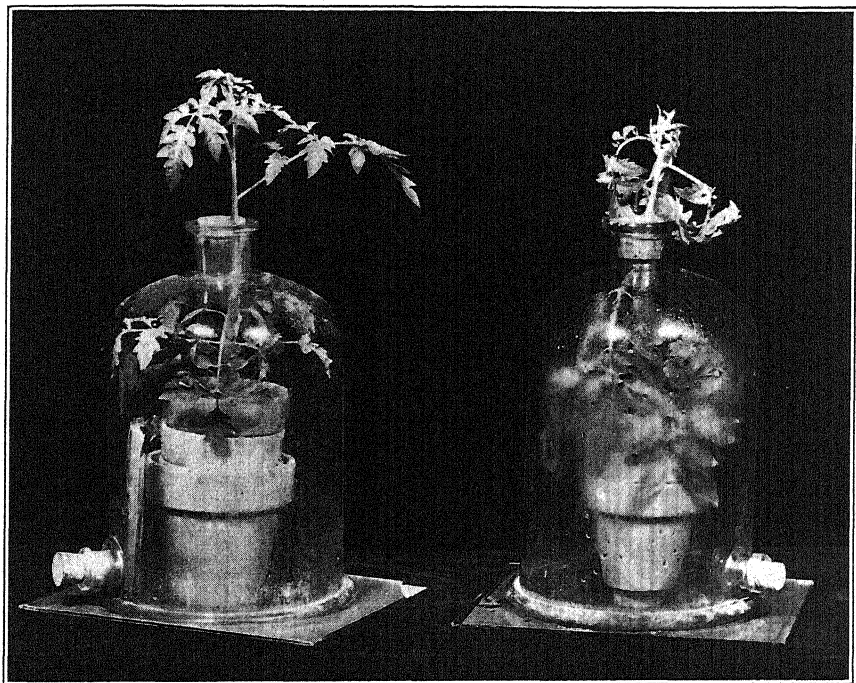


FIGURE 2. Left: Tomato plant with its lower part sealed in a bell jar containing air. Right: Tomato plant with its lower part sealed in a bell jar containing a mixture of air and illuminating gas. The upper part of the plant shows a typical ethylene response. This shows that the gas from within the bell jar passed into and throughout the entire plant.

gas traveled from the upper petioles downward, from the middle petioles in both directions, and from the lower petioles upward. Even tips of long leaves 15 inches or more away from the treated petioles showed epinasty. The results, then, showed that the gas moved in through the petiole, up and down through the stem, and out through the petiole and leaves. In other words, the gas moved in all directions through the plant. Additional experiments were performed in order to determine whether or not the gas gained entrance only through the cut surfaces made by severing the leaflets.



Tall tomato plants were placed so that the tops protruded through the upper opening of bell jars while the lower portions were sealed in bell jars containing gas (Fig. 2). After a few hours in this treatment the top as well



FIGURE 3. Left: Tomato plant with its top sealed in a flask containing air. Right: Tomato plant with its top sealed in a flask containing a mixture of gas and air. A  $1\frac{1}{2}$  inch region of the stem had been killed by an electric current before the treatment began. The gas traversed the dead portion as readily as living tissue.

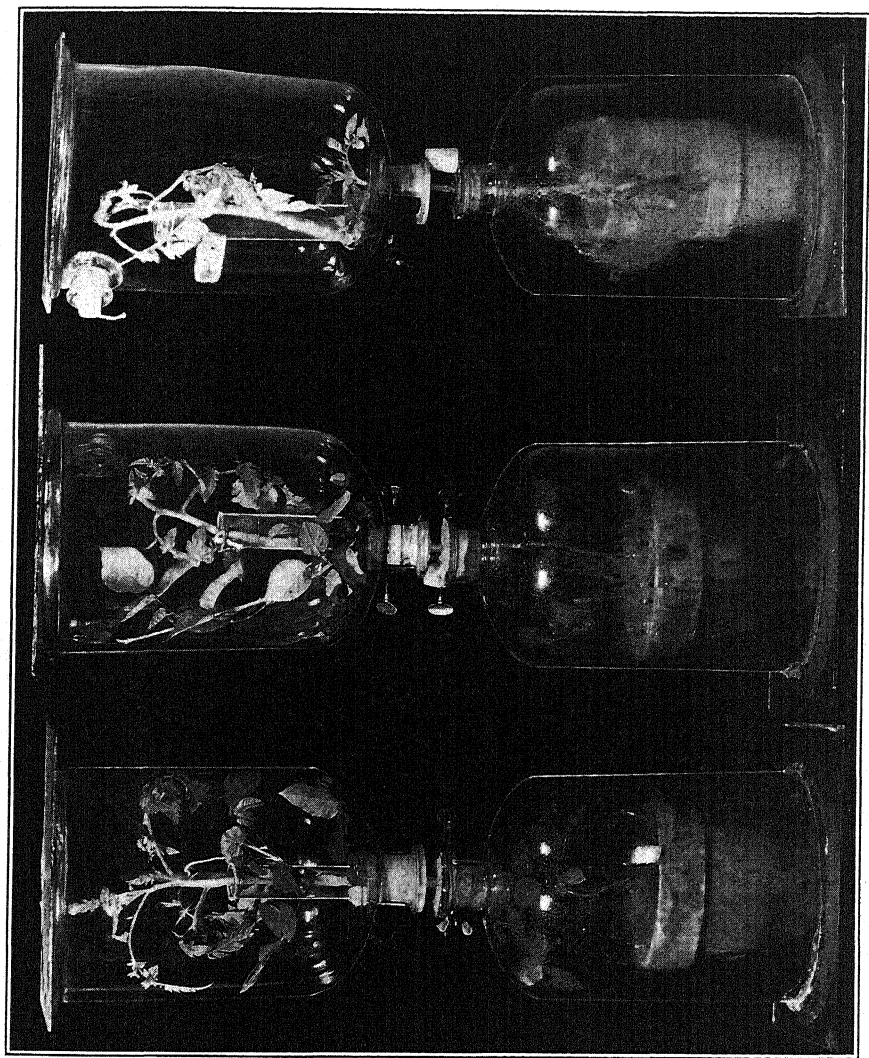


FIGURE 4. Left: Rose plant with its lower part sealed in a bell jar containing air. Top part sealed in inverted bell jar together with a tomato cutting in air. Middle: Rose plant same as one on the left but having gas in the lower bell jar instead of air. The leaves of the rose plant and the cutting in the upper bell jar showed the ethylene response. Right: Tomato plant treated as described for the rose plant shown in middle. Note the response of tomato cutting in the upper bell jar as shown by the downward bending of the petioles near the place where they are attached to the stem.

as the lower portion showed the ethylene response. In a similar experiment where there were no cut surfaces exposed, the top was enclosed in a flask with gas (Fig. 3). With this method all of the leaves below the flask responded. The plant in the photograph shows a  $1\frac{1}{2}$  inch region of the stem which had been previously killed by a 30 second exposure to 320 volts of electricity. The killed part was allowed to dry and shrivel before the treatment started, but the dead section of the stem did not prevent the downward movement of the gas.

Figure 4 shows the final method of procedure where roses and tomatoes were used to show that the gas passed through the plant tissues and that the response was not due to leakage from the gas chamber. The lower part of the plant was sealed in a bell jar containing gas, while the top was sealed in a bell jar containing a tomato cutting but no gas. A portion of the stem between the bell jars was exposed to outside air to prevent any possible chance of gas leaking into the upper jar. Within a 24 hour period the top of the plant and the tomato cutting responded to gas. The response of the tomato cutting indicated the presence of gas in the upper bell jar since it was in every way separated from the plant which had its lower part in gas. The gas must have traveled through the stem to the top and then out through the leaves. There are stomata in the epidermis of the stem, the petiole, and the leaflets. The number varies greatly with the position of the epidermis but with several counts it was estimated that the leaf blade has 10 to 20 times as many per unit area as the stem or petiole. The entrance of gas might not be limited to stomata.

Six other species (*Rosa* [hybrid tea] var. Madame Butterfly, *Pelargonium hortorum* Bailey var. Alphonse Ricard, *Lilium longiflorum* Thunb. var. *giganteum*, *Lilium speciosum* Thunb. var. *rubrum*, *Hedera helix* L., and *Nephrolepis exaltata* Schott. var. *bostoniensis* form Whitmanii) were used in experiments with the method shown in Figure 4 and found as effective as the tomato in permitting the transfer of gas. In the case of the rose, the leaves enclosed in the upper bell jar as well as those in the lower jar showed the characteristic yellowing along the veins in addition to the fall of leaflets, indicating the presence of gas throughout the plant, though only the lower part had been treated. The fact that the tomato cutting enclosed with the upper portion of the rose responded within a 24 hour period indicated a fairly rapid movement of the gas through stems and leaves of even woody plants.

Ferns treated according to the method shown in Figure 4 gave no indication of the presence of gas, but the tomato cutting enclosed in the upper bell jar with the tops of the fronds responded to ethylene within a 24 hour period. This is a case in which a resistant species allows the gas to pass readily through its tissue without injury. Similarly, English ivy was not injured though the gas traveled through its leaves and stem. Lilies and

geranium transmitted the gas but the leaves turned slightly yellow, characteristic of both species for certain concentrations of the gas.

Various concentrations of ethylene or illuminating gas ranging from 1 to 50 per cent were used to produce the responses reported in this paper. The effects were approximately the same for all concentrations used.

#### DISCUSSION

The results of these experiments show that ethylene gas enters and moves fairly rapidly through the tissues of plants. The quantity entering, the places of entrance, and the tissues permitting its passage up and down the stem are still unknown. Is it dissolved in the cell sap or does it travel through the intercellular spaces?

The presence of the gas is readily detected by the tomato plant but since it responds alike over a wide range of concentrations there is no good method of determining the amount of gas present. There is an indication that the gas concentration was fairly high since approximately 10 times as much gas is necessary to affect the rose as is required to cause a response in the tomato. There are no chemical methods for detecting such low concentrations of ethylene in the atmosphere.

The results of the experiments show that the gas can enter the plant through the epidermis of the stem, petiole, or leaf blade. All of these structures have stomata but the blade has 10 to 20 times as many as the other organs. Stomata are said to close at night but susceptible plants respond to ethylene at night as well as during the day. Some stomates probably remain open at night and it is likely that none of them closes tightly enough to completely shut out ethylene.

The results of the experiments show that the gas can move in all directions from the point of entrance, but there is no clue as to whether it diffuses through the intercellular spaces or whether it is carried in the transpiration stream. Since ethylene is only slightly soluble in water, it could hardly travel in the cell sap as rapidly as indicated by the results. That living cells are not necessary is shown by the fact that the gas was able to traverse a 1 1/2 inch dead portion of the stem. Although the stem shriveled it still conducted sufficient water to keep the plant turgid. Probably the gas moves largely through intercellular spaces but perhaps to some extent in the cell sap.

Since ethylene passes through plant tissue so readily it would seem that other gases might act similarly. If so, the plant has an effective aerating system. Cannon (1) at one time could not account for all of the oxygen that was found in the container holding nitrogen around the roots and suggested that it might be due to oxygen diffusing in from the air through the stems. In the light of the data reported in this paper that would seem a logical conclusion.

## SUMMARY

1. Ethylene gas may enter through the stem, petiole, or leaf blade and spread throughout the entire plant.
2. Plants with their lower portions sealed in bell jars containing gas and their tops sealed in inverted bell jars containing tomato cuttings but no gas, transmitted enough of the gas to cause the ethylene response of the tomato cuttings.
3. Ethylene traversed a dead region of a tomato stem causing the leaves beyond to respond as if enclosed with gas.
4. The seven species tested (*Lycopersicon esculentum* Mill. var. Bonny Best, *Rosa* [hybrid tea] var. Madame Butterfly, *Hedera helix* L., *Nephrolepis exaltata* Schott. var. *bostoniensis* form *Whitmanii*, *Lilium longiflorum* Thunb. var. *giganteum*, *Lilium speciosum* Thunb. var. *rubrum*, and *Pelargonium hortorum* Bailey var. Alphonse Ricard) were found to be permeable, allowing the gas to enter at any region and to diffuse through and out of the plant. The Boston fern and *Hedera helix* were not affected in spite of the fact that the gas traversed the entire plant.
5. These facts show the effectiveness of the aeration system in allowing the transport of gases throughout the tissues of plants.

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# THE EFFECT OF TREATMENTS WITH ETHYLENE CHLORHYDRIN ON THE pH OF THE EXPRESSED JUICE OF POTATO TUBERS<sup>1</sup>

LAWRENCE P. MILLER

In connection with a study of the enzyme activities of juices from potatoes (*Solanum tuberosum* L.) treated with chemicals (6), it was noted that treatments with ethylene chlorhydrin changed the pH value of the juice in an alkaline direction, the difference between treated and check lots sometimes being as much as a whole pH unit. This pH difference in the chlorhydrin-treated potatoes is especially interesting in view of the fact that although sodium thiocyanate and thiourea are effective in breaking dormancy, treatment with these chemicals has only a slight effect on the pH of the expressed juices. The change necessary to cause a pH shift of from 0.5 to 1.0 unit must be extensive since potato juice is strongly buffered.

In this paper are presented the results obtained thus far in connection with a detailed study of this effect on the pH value of the juices.

## METHODS

*Treatments.* The methods used in treating the potatoes were those described by Denny (5). In the "dip method" the tubers were cut into pieces with one eye each and weighing about one ounce, and the pieces were dipped into a solution of ethylene chlorhydrin of the strength shown in the tables and then stored in a closed container for 24 hours. (If a different storage period was used this is noted in the tables.) At the end of this storage period the pieces were planted in soil in flats where they were kept until it was desired to obtain samples of the juices. The checks consisted of pieces from the same lot of tubers that had been handled in exactly the same way as the treated lots except that they were dipped into water instead of ethylene chlorhydrin solutions. The vapor treatments were made by placing the tubers in glass or earthenware vessels with covers, in which they were exposed for 24 hours (if a shorter period was used this is shown in the tables) to ethylene chlorhydrin vapor evaporating from pieces of cheesecloth placed loosely at the top of the container. Check tubers were placed in similar containers containing no chlorhydrin. After treatment the intact tubers were stored in paper bags until samples were required.

*Sampling.* To obtain samples of juice the pieces were washed free of dirt, dried with cheesecloth, the peel and callous tissue removed, and the pieces then either pounded in a mortar and the juice pressed out through cheesecloth or the material was ground through a food grinder and then

<sup>1</sup> Herman Frasch Foundation for Research in Agricultural Chemistry, Paper No. 23.

squeezed through cheesecloth. For the purpose of these experiments there was no essential difference between the juice obtained by the two methods, the method chosen being merely a matter of convenience, the food grinder being used in the case of large samples. In a given series, however, the treated and check juices were, of course, always obtained in the same manner. When the juice was obtained from whole tubers, similar methods were used; in some cases the peel was included in the sample as indicated in some of the experiments described.

*pH determinations.* The pH determinations, unless otherwise stated, were made with the quinhydrone electrode, using a saturated calomel half-cell as the reference electrode. The apparatus was frequently checked against M/20 potassium acid phthalate and other buffers. Determinations were also made with the glass and hydrogen electrodes as shown below. To a limited extent tests were made colorimetrically. In some instances the differences between treated and check juices were large enough to be shown by litmus paper.

## RESULTS

### PH OF TREATED AND CHECK JUICES

#### *With the Quinhydrone Electrode*

The results of treatments with various concentrations of ethylene chlorhydrin by both the vapor and dip methods on the pH of the juices obtained four days after treatment as determined by the quinhydrone electrode are shown in Table I. It is seen that the change may be as much as a whole pH unit, and that the weaker treatments resulted in less of a pH change than the stronger ones.

TABLE I

EFFECT OF TREATMENTS WITH ETHYLENE CHLORHYDRIN ON THE PH OF THE EXPRESSED JUICE OF POTATOES

Treatment	Concentrations	Quinhydrone pH
Whole tuber, vapor	1 cc. per liter for 24 hrs.	6.78
	" " " " " 12 "	6.51
	" " " " " 6 "	6.59
	Check	6.00
Cut pieces, dip	100 cc. per liter stored 24 hrs.	7.35
	" " " " " 12 "	7.32
	" " " " " 6 "	6.81
	Check	6.00

It has previously been reported (6) that juices from potatoes treated with ethylene chlorhydrin are higher in reducing substances (substances which react with iodine in acid solution, and which decolorize methylene blue) than check juices. The successful use of the quinhydrone electrode

for pH determination depends upon the maintenance of a definite ratio between quinone and hydroquinone (4, p. 404-417), and it is necessary to avoid the presence of substances which would change this ratio. But it has been shown by Biilmann (2) that the quinhydrone electrode can be used for the determination of the pH of dilute nitric acid solutions and solutions of unsaturated organic acids, solutions which Clark (4) states cannot well be handled with the hydrogen electrode. In the case of the potato juices in question it was quite easy to find out whether the reducing substances were a factor in the observed pH difference by a determination of the pH of the treated and check juices after aeration, since it has been found that after aeration there is no longer any difference in the reducing substances mentioned above between the treated and check juices (6). Many such determinations gave results such as are illustrated in Table II. It is seen, then,

TABLE II  
EFFECT OF AERATION AND BOILING ON THE PH OF TREATED AND CHECK JUICES

Treatment	pH		
	Expressed juice	Aerated juice	Boiled filtered juice
Whole tuber, vapor 1 cc. per liter	6.64	7.00	7.05
Check	6.25	6.41	6.41

that after aeration, when the reducing substances are no longer a factor, the pH difference persists; in fact, it is a little larger than before aeration. The probable explanation of this fact will be given later in the paper. When the juice is boiled and filtered the same pH values are obtained as for aerated juice, although this procedure does not destroy the substances which react with iodine in acid solution. On the basis of this evidence it was, therefore, concluded that the reducing substances present in the juice from treated potatoes did not interfere in the quinhydrone determinations. This conclusion is, of course, also fortified by determinations made with other electrodes as shown below.

#### *With Other Electrodes*

In Table III are shown some determinations made with the glass and hydrogen electrodes as compared with the quinhydrone electrode. Similar differences between treated and check juices are given by these electrodes. Results obtained with the hydrogen electrode, however, are not considered satisfactory since values depended on the length of time hydrogen was bubbled through the juice, the values becoming greater the longer the bubbling was continued, finally reaching more or less of a maximum. At this point there is always a difference between treated and checks as seen in Table



III. These high values are, however, in all probability incorrect, hydrogen apparently having some action upon the juice. This point was not further investigated, but the high values shown for the hydrogen electrode in

TABLE III  
pH OF TREATED AND CHECK JUICES DETERMINED WITH VARIOUS ELECTRODES

Treatment (whole tuber, vapor)	pH		
	Quinhydrone electrode	Glass electrode	Hydrogen electrode
1 cc. per liter, 24 hrs.	6.73	6.62	7.29
1/5 " " " " "	6.61	6.45	6.99
Check	6.39	6.16	6.66

Table III were also given by the quinhydrone electrode if hydrogen was first bubbled through the juices.

#### *With Indicators*

Differences in pH could also be observed by placing bromthymol blue or phenol red on slices of treated and check tubers. In some cases the pH differences of the juices were large enough so that differences could be detected when litmus paper was used as the indicator.

#### DIRECT EFFECT OF ETHYLENE CHLORHYDRIN

It was necessary to determine whether ethylene chlorhydrin could cause this pH change through any direct effect. Solutions of ethylene chlorhydrin are always slightly acid due to the presence of small quantities of hydrochloric acid resulting from hydrolysis (3). One would not expect, therefore, that ethylene chlorhydrin could increase the alkalinity of the

TABLE IV  
pH VALUES RESULTING WHEN ETHYLENE CHLORHYDRIN IS ADDED DIRECTLY TO THE JUICE OR TO A M/15 PHOSPHATE BUFFER

Anhydrous chlorhydrin, g. per 100 cc.	Juice from untreated potatoes	Juice from treated potatoes*	M/15 phosphate buffer
0	6.44	7.00	6.59
0.4	6.39	6.98	6.54
0.8	6.39	7.02	6.54
1.2	6.39	7.00	6.54
1.6	6.39	6.96	6.53
2.0	6.36	6.98	6.46
4.0	6.31	6.73	6.37
8.0	6.17		6.14
12.0	6.05		5.97
14.0	5.95		5.29
20.0	5.88		

\* These potatoes had been treated 5 days previously with ethylene chlorhydrin vapor, 1 cc. per liter.

juices by any direct action. This could only be possible through secondary reactions of some kind. To test this point, however, various amounts of ethylene chlorhydrin were added directly to a treated and a check juice and to a phosphate buffer. Results in Table IV show that the first effect on the solutions was to make them more acid and this effect did not take place until the concentration reached about four grams per 100 cc. of solution. Similar results were obtained when the juices were boiled in the presence of various amounts of ethylene chlorhydrin and when they stood with ethylene chlorhydrin at room temperature under toluene for 24 hours. It follows then that the increase in pH resulting from treatment with ethylene chlorhydrin is not due to any direct chemical reaction between ethylene chlorhydrin and the juices but must be the result of some tissue response to this chemical.

#### DISSOLVED CARBON DIOXIDE OF THE JUICES

It will be recalled from Table II that when the freshly expressed juices are aerated or boiled and filtered there is an increase in their pH value, the increase being slightly greater in the treated than in the check lots. It had also been noted that on standing, the pH of the juice did not remain constant but increased slowly to a fixed value. The fact that about the same pH was reached whether the juices were aerated or boiled indicated the possibility of a dissolved gas. Since the change was toward the alkaline side, dissolved  $\text{CO}_2$  was determined. Accordingly,  $\text{CO}_2$  free air was passed through the juices and then through  $\text{Ba}(\text{OH})_2$  in a Van Slyke-Cullen apparatus (11). After several hours the  $\text{Ba}(\text{OH})_2$  was filtered, the precipitated  $\text{BaCO}_3$  washed and dissolved in 25 cc. N/10 HCl, and the amount of  $\text{CO}_2$  determined by back titration with N/10 NaOH. In Table V are shown the

TABLE V  
CARBON DIOXIDE GIVEN OFF BY THE EXPRESSED JUICES ON AERATION

Variety	Concentration of dip treatment	Days after treatment	pH freshly expressed juice	Mg. $\text{CO}_2$ given off by 20 cc. juice	Mg. $\text{CO}_2$ given off by 20 cc. acidified juice*	pH of boiled filtered juice
Irish Cobbler	70 cc. per liter	6	6.85	15.66	14.08	7.05
	25 " " "		6.71	14.12	13.64	6.88
	Check		6.27	9.76	9.24	6.39
Bliss Triumph	70 cc. per liter	7	6.61	14.04	—	6.73
	25 " " "		6.49	12.39	10.96	6.61
	5 " " "		6.17	10.63	8.10	6.27
	Check		6.14	9.53	6.34	6.17
Early Ohio	60 cc. per liter	3	6.29	14.40	—	6.58
	Check		6.07	10.21	—	6.24

\* 1 1/2 cc. 50 per cent  $\text{H}_2\text{SO}_4$  added to 20 cc. juice.

results of such determinations made with three varieties of potatoes treated with various concentrations of ethylene chlorhydrin. The determinations were made from three to seven days after treatment. The  $\text{CO}_2$  given off is greater in the treated than the check lots, the amounts given off forming a series in line with the series of concentrations used. The addition of one and one-half cc. of 50 per cent  $\text{H}_2\text{SO}_4$  to 20 cc. of the juice before aeration did not appreciably alter the result, indicating that most of the  $\text{CO}_2$  given off came from dissolved  $\text{CO}_2$  rather than from any such reaction as that of tyrosinase, for example, in which  $\text{CO}_2$  is one of the products (1). On the basis of the  $\text{CO}_2$  content alone one should expect the treated to be more acid than the check.

The data of Table V show the reason for the pH changes resulting in the juices on standing and account for the greater change in the treated than in the check lots. The increased  $\text{CO}_2$  content of the treated juices is interesting in view of the work of Smith (9) who found that treatment with ethylene chlorhydrin increased respiration.

#### TIME RELATIONS IN EFFECT OF ETHYLENE CHLORHYDRIN ON PH

##### *A Study of the pH of Check Tubers*

*Variation of individual potatoes.* Preliminary to a rather detailed study of the time relations involved in the pH change resulting from ethylene chlorhydrin treatments, it was necessary to determine the extent of the variation of the pH of the lots of potatoes to be used. The potatoes used in

TABLE VI  
VARIATIONS IN THE PH OF INDIVIDUAL POTATOES

Variety	No. of tubers tested	Maximum value	Minimum value	Average	P.E. single determination	P.E. average of two tubers
Irish Cobbler	16	5.88	5.63	5.81	$\pm 0.048$	$\pm 0.034$
Bliss Triumph	13	5.90	5.65	5.80	$\pm 0.054$	$\pm 0.038$
Early Ohio	15	6.00	5.68	5.84	$\pm 0.061$	$\pm 0.043$

these experiments were Irish Cobbler, Bliss Triumph, and Early Ohio varieties which had been grown in the Institute gardens. Determinations of the pH values of the expressed juice of individual potatoes, skin included, were made. These data are summarized in Table VI. An examination of the values for the probable errors shows that the variation among the individual potatoes is rather small in all three varieties and that if samples of only two tubers are used, a pH difference between them of about 0.15 pH is significant.

*pH of juices from different parts of the potato.* Although there are many reports in the literature of pH determinations on potato tubers no one seems to have studied the hydrogen ion concentration of various regions of the tubers, although Samuel (8, p. 399) without giving data states that "a hydrogen ion concentration map of a potato tuber could readily be made out, the reaction becoming slightly more acid as one proceeded outwards from the center, the vascular circle and the tip being most acid." Whether the tubers tested by Samuel were dormant is not given in his paper.

TABLE VII  
pH OF VARIOUS LAYERS OF THE POTATO TUBER

Part of potato	Variety	
	Irish Cobbler	Early Ohio
Outside	1	5.65
	2	5.85
	3	5.93
	4	6.05
	5	6.10
	6	6.15
	7	6.22
	8	6.25
Inside		

In the case of the dormant tubers used for our experiments it was found that if successive layers are peeled from the outside and the pH of the expressed juices determined, values as shown in Table VII are obtained. There is a gradual decrease in hydrogen ion concentration toward the center of the potato, each successive layer giving a value slightly more alkaline until the center is reached which is the most alkaline of all. The pH of the center is about one-half a pH unit higher than that of the peel.

It does not follow, of course, that non-dormant tubers show the same pH picture. In fact tubers from these lots when examined months later no longer showed these differences between the various layers, the average pH for any layer now being 6.1 or 6.2. Table VI shows that these tubers had pH values of about 5.8 when freshly dug. Whether there is any direct correlation between this rise in pH during storage and the end of the dormant period has not been determined.

#### *A Study of the pH of Treated Tubers*

*Vapor treatments.* Whole tubers of Bliss Triumph, Early Ohio, and Irish Cobbler varieties were given vapor treatments of one cc. of 40 per cent ethylene chlorhydrin per liter for 24 hours and pH determinations made of various parts of these tubers at intervals after treatment. Similar determinations were made on check tubers at the same time. For these experiments the tubers were cut into four layers and juices from two tubers

TABLE VIII  
TIME RELATIONS OF EFFECT OF ETHYLENE CHLORHYDRIN ON pH OF POTATOES

Variety	Part of potato	Hours after treatment											
		0		24		48		120		504		Check	Check
		Treated	Check	Treated	Check	Treated	Check	Treated	Check	Treated	Check		
Bliss Triumph	Outside 1	6.36	5.76	6.56	5.80	6.61	5.85	6.39	5.88	6.00	5.71		
	2	6.29	6.02	6.44	5.93	6.73	5.92	6.64	5.90	6.10	5.76		
	3	6.32	6.09	6.34	6.14	6.88	6.09	6.71	6.05	6.19	5.85		
	Inside 4	6.31	6.12	6.22	6.22	6.63	6.09	6.70	6.12	6.22	5.88		
Early Ohio	Outside 1	6.36	5.76	6.41	5.76	6.42	5.87	6.37	5.85	6.31	5.90		
	2	6.27	5.87	6.49	5.93	6.39	6.03	6.44	5.93	6.31	5.90		
	3	6.20	6.14	6.47	6.07	6.56	6.14	6.47	6.09	6.22	5.98		
	Inside 4	6.27	6.25	6.39	6.27	6.51	6.27	6.44	6.05	6.39	6.29		
Irish Cobbler	Outside 1	6.61	5.71	6.64	5.71	6.61	5.85	6.63	5.87	6.36	5.85		
	2	6.39	5.97	6.85	5.90	6.66	6.03	6.88	6.05	6.19	6.00		
	3	6.29	6.08	6.70	5.97	6.56	6.09	6.78	6.05	6.36	6.00		
	Inside 4	6.27	6.31	6.42	6.15	6.39	6.17	6.54	6.24	6.27	6.00		

mixed for pH determinations of each layer. As seen in Table VIII an appreciable increase in pH is evident in the first two layers and perhaps in some cases in the third layer by the time the treatment is ended. The data for succeeding time intervals show how the pH change gradually becomes manifest even in the center of the potato, where it does not occur, however, until about 48 hours after the end of the treatment. The highest values are found for the second and third layers, this being due chiefly to the fact that the peel layer is considerably more acid than the succeeding layers, the change in pH resulting from the treatment being much the same for all three layers. Figures obtained after 504 hours show that after such a long time has elapsed, the pH values of the treated tubers had fallen to some extent but were still higher than the checks.

TABLE IX  
TIME RELATIONS IN EFFECT OF ETHYLENE CHLORHYDRIN DIP TREATMENT  
ON pH OF DORMANT TUBERS

Variety	Treatment	Hours after end of treatment		
		30	56	80
Bliss Triumph	60 cc. per liter, 16 hrs.	6.47	6.59	6.47
	Check	6.05	6.00	5.97
Irish Cobbler	60 cc. per liter, 16 hrs.	6.47	6.61	6.56
	Check	6.10	6.00	5.97

*Dip treatments.* In Table IX are shown some pH values obtained at intervals after the dip treatments. These determinations were made on the juices from the pieces after the peel and callus had been removed. It is seen that as soon as 30 hours after the end of the treatments there is a considerable pH difference between treated and check.

#### EFFECT OF TREATING DIFFERENT PARTS OF THE TUBERS SEPARATELY

Since the pH rise apparently took place first in the outer portions of the tubers, the question arises whether the ethylene chlorhydrin first acts on the bud tissue and starts some processes which later make themselves felt throughout the whole tuber. As far as pH is concerned this could easily be determined by first cutting up the tubers into four layers and treating these separately. Table X shows that at the end of the treatment the pH change had taken place in all four layers of the tuber. The potato tuber need not be intact for these pH changes to result from the ethylene chlorhydrin treatments.

#### RELATIONS BETWEEN DORMANCY AND THE PH CHANGE

The question arises whether the response of the tubers to ethylene chlorhydrin is characteristic of dormant potatoes only or whether non-



dormant tubers will also change in pH when subjected to ethylene chlorhydrin vapors. Experiments have shown that when non-dormant tubers are given the vapor treatment, the pH change produced is much less than that resulting from the treatment of dormant tubers.

TABLE XI  
TIME RELATIONS IN EFFECT OF ETHYLENE CHLORHYDRIN TREATMENTS ON pH  
OF NON-DORMANT POTATOES

Variety	Treatment	Hours after end of treatment					
		24	48	72	120	144	168
Early Ohio	60 cc. per liter, dip Check	6.02	6.25	6.29		6.31	6.53
		6.00	5.98	6.00		6.03	6.19
Bliss Triumph	70 cc. per liter, dip		6.14	6.24	6.46		6.76
	25 cc. per liter, dip		6.09	6.20	6.42		6.51
	5 cc. per liter, dip		6.07	6.03	6.14		6.10
	Check		6.00	5.93	5.93		6.03
Irish Cobbler	70 cc. per liter, dip		6.41	6.42	6.75		6.66
	25 cc. per liter, dip		6.31	6.41	6.49		6.68
	5 cc. per liter, dip		6.17	6.03	6.42		6.32
	Check		6.07	6.05	6.07		6.14
Bliss Triumph	1 cc. per liter, vapor Check		6.12				6.31
			6.22				6.00
Irish Cobbler	1 cc. per liter, vapor Check		6.05				6.36
			6.12				6.02

Values in Table XI (vapor treatments) are typical of the results obtained. The potatoes were from the same lot as those used for the data of Table VIII but the treatments were made about five months later. The pH determinations were made on juice from the whole tubers, minus the peel. The pH change produced is only from 0.2 to 0.3 of a unit and this change takes place more slowly than the change resulting from the treatment of dormant tubers.

On the other hand when dip treatments are used pH differences of as much as 0.5 to 0.75 of a pH unit may result from the treatment of non-dormant tubers. But the data in Table XI show that the maximum change does not occur until five days after treatment in the case of Irish Cobbler and not until seven days in the case of Early Ohio and Bliss Triumph varieties. When the tubers are dormant this change takes place more quickly, as was brought out in a previous publication (6) where samples were taken three or four days after treatment, before any visible sprouts were produced. This is also shown in Table IX.

#### EFFECT ON THE BUFFERS OF THE JUICES

The buffers of the expressed juice of potato tubers have been studied by Ingold (7). He gives figures for the buffering capacity of the juices of six



varieties of potatoes in the range from pH 4 to pH 7, expressed in terms of the buffer index values as used by Van Slyke (10), where  $\beta$ , the buffer index, is derived from the expression  $\beta = dB/dpH$ , where  $dB$  equals the number of gram equivalents of acid or alkali necessary to bring about a certain change in pH in one liter of solution, and  $dpH$  is the change in pH produced.

TABLE XII  
BUFFER INDICES OF JUICES OF POTATO VARIETIES USED

Variety	Van Slyke buffer indices				
	pH 3-4	pH 4-5	pH 5-6	pH 6-7	pH 7-8
Spaulding Rose	0.0465	0.0365	0.023	0.016	0.0145
Irish Cobbler	0.054	0.047	0.0245	0.0175	0.015
Bliss Triumph	0.0675	0.0495	0.031	0.020	0.019
Early Ohio	0.071	0.051	0.036	0.0265	0.0195

The varieties of potatoes used in our experiments were more highly buffered than those reported upon by Ingold. Table XII gives the buffer index values at intervals of one pH unit from pH 3 to pH 8 for Spaulding Rose, Irish Cobbler, Bliss Triumph, and Early Ohio varieties. The titration

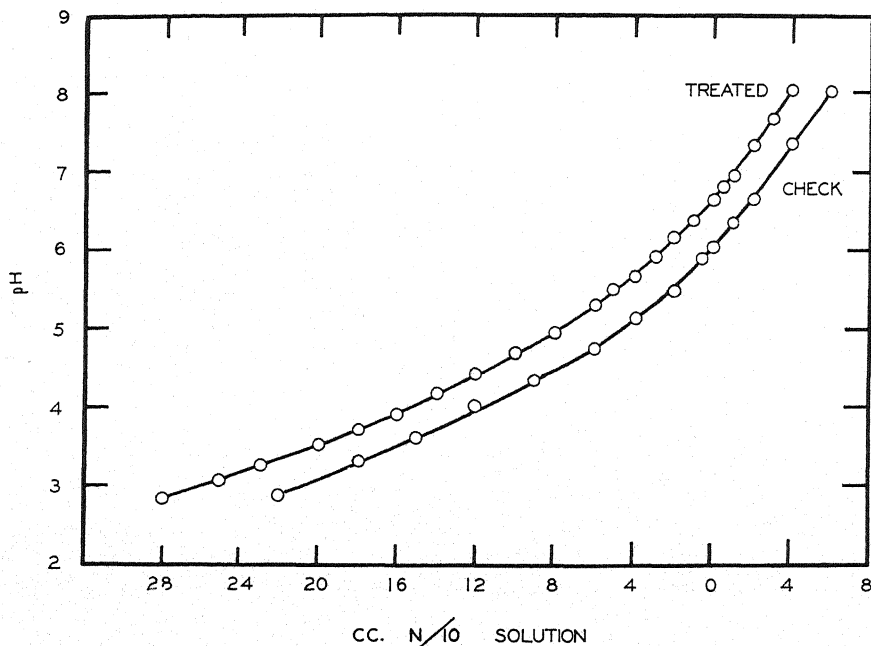


FIGURE 1. Titration curves of treated and check potatoes, Spaulding Rose variety, vapor treatment, 1 cc. per liter.

curves of these juices from which the buffer indices were calculated show that for 20 cc. of juice from 1.5 to 2.0 cc. N/10 NaOH are necessary to cause a change in pH from 6.0 to 6.5.

Titration curves for treated and check juices as given in Figure 1 show that ethylene chlorhydrin treatments bring about this pH change without any pronounced disturbance in the buffers of the juices. The upper curve in Figure 1 represents the titration of juice from potatoes which had undergone a vapor treatment of one cc. per liter for 24 hours, and the lower curve represents the corresponding check juice. Many such titration curves have been made for all the varieties used in this work and in general they exhibit the same form as shown in Figure 1, except that in some cases there is tendency for the two curves to be somewhat closer together in the acid range. However, a study of such curves together with the buffer indices calculated from them has failed to show any consistent buffer difference between treated and check that could not be ascribed to experimental error.

#### EFFECT OF ETHYLENE BROMOHYDRIN

Preliminary tests indicate that treatment with ethylene bromohydrin produces a pH change of the same order as treatment with the chlorhydrin. In a particular case, vapor treatment with one cc. per liter caused an increase in the pH of the juice four days after treatment as follows: Irish Cobbler, 0.86; Early Ohio, 0.67; Bliss Triumph, 0.95.

#### SUMMARY

1. The treatment of dormant potatoes with ethylene chlorhydrin invariably results in an increase in the pH value of the expressed juice; this increase usually equals from 0.5 to 0.75 of a pH unit, but may be as much as 1.0. This pH difference has been demonstrated with the use of the quinhydrone, glass, and hydrogen electrodes and with indicators. The value obtained with the hydrogen electrode depends upon the length of time hydrogen is bubbled through the solution, the pH value increasing with time to a value considerably higher than that given by the quinhydrone electrode; however, the pH difference between treated and check persists.

2. The addition of various amounts of ethylene chlorhydrin directly to potato juice or phosphate buffers produces no change in pH until a concentration of about four grams per 100 cc. is reached when it begins to cause a slight shift toward the acid side. The pH changes produced by ethylene chlorhydrin treatments, therefore, can not be due to any direct effect but are the result of some tissue response to treatment with this chemical.

3. The potato juices as expressed contain considerable quantities of CO<sub>2</sub> which are given off on aeration; treated juices giving off about 15 mg.

CO<sub>2</sub> per 20 cc. juice, and check juice about 10 mg. It is believed that this accounts for the increase in pH resulting when these juices are boiled or aerated, the treated showing a slightly higher increase than the check. The addition of acid to the juice before aeration does not increase the amount of CO<sub>2</sub> given off.

4. A determination of the variation of the pH of individual potatoes of Irish Cobbler, Bliss Triumph, and Early Ohio varieties used for the study of the time relations in the effect of ethylene chlorhydrin on the pH, shows that these lots of potatoes were quite uniform, the probable error of a single pH determination being as follows: Irish Cobbler,  $\pm 0.048$ ; Bliss Triumph,  $\pm 0.054$ ; Early Ohio,  $\pm 0.061$ .

5. An examination of different layers of the dormant tubers in the case of all varieties studied (Irish Cobbler, Bliss Triumph, Early Ohio, and Spaulding Rose) shows that there is a progressive increase in the pH values of the expressed juice of the tissues from the outer portions to the inside of the tuber, the pH of the center in some cases being 0.5 pH higher than the peel.

6. A study of the time relations in connection with the pH change produced by ethylene chlorhydrin treatments shows that in the case of vapor treatments of 24 hours duration, the pH of the outer layers has shifted toward the alkaline side by the time the treatment is ended. If the pH is determined in the various portions at intervals after treatment, it is possible to follow the progress of the pH change toward the center of the potato. The maximum change produced is evident about 48 hours after the end of the treatment. The highest values are found for the second and third layers; this is due to the fact that the peel layer is considerably more acid than the succeeding layers, the absolute change resulting from the treatment being much the same for all three layers. The change produced in the fourth portion or center of the tubers is not as large.

7. The presence of eyes is not necessary for the treatment to induce this pH change; various parts of the potato treated separately show the pH increase.

8. This increase in pH is not produced as readily in non-dormant tubers, vapor treatments result only in changes of a few tenths of a pH. Dip treatments will induce changes comparable to those produced with dormant potatoes, but the increase in pH does not result as quickly after treatment as with dormant tubers.

9. Buffer index values are given for juices from Irish Cobbler, Bliss Triumph, Early Ohio, and Spaulding Rose varieties. To cause a change in the pH of these juices from 6.0 to 6.5, 1.5 to 2.0 cc. of N/10 NaOH for each 20 cc. of juice are necessary.

10. An examination of the titration curves of treated and check juices

of all varieties used has failed to bring out any consistent differences in the buffers between the treated and check juices.

11. Preliminary experiments with ethylene bromohydrin show that it produces a pH change comparable to that produced by the chlorhydrin.

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## THE ORIGIN OF ADVENTITIOUS ROOTS IN CUTTINGS OF *PORTULACA OLERACEA* L.

MARY H. CONNARD AND P. W. ZIMMERMAN

In stem cuttings the place where roots emerge varies with the species. It might be assumed, therefore, that some internal differences also occur. This may be one reason why botanists are not entirely agreed as to the exact internal origin of adventitious roots from stem cuttings. In this paper the authors will not discuss the general subject of the origin of these roots but will set forth the situation as it appears in a specific case, namely in cuttings of *Portulaca oleracea* L. For that reason a long review of the literature will not be made. It will suffice to refer to only two papers. In 1888 Tieghem and Douliot (2, pp. 409 and 488) concluded that endogenous adventitious roots occurring naturally in young portions of stems originated entirely in the pericycle. They state that in older portions of the stems, or later in ontogeny, roots might arise in phloem parenchyma, or still later in the cambium.

In 1929 Priestley and Swingle (1, p. 64), after reviewing the literature, wrote, "The points that should again be emphasized are the pericyclic origin of roots in young stems, and the origin in the neighborhood of the cambium in older stems; in either case the roots are generally intimately associated with the rays; furthermore, their origin involves more than one layer of cells."

### MATERIALS AND METHODS

The material used consisted of cuttings of purslane (*Portulaca oleracea*) collected from the gardens of Boyce Thompson Institute. The cuttings were made at various times from July to September and placed in water or sand media to permit of root growth. Properly selected cuttings made in July rooted uniformly in four to six days. The roots arose only at the base of the cutting and emerged through the cut surface parallel to the main axis of the stem (Fig. 1). To obtain sections with root primordia, basal pieces of the stems about 5 mm. long were cut at intervals beginning 40 hours after the cutting was planted and killed in chrom-acetic solution. These pieces were embedded in paraffin and then cut into serial sections 12 to 18  $\mu$  in thickness. Safranin and Delafield's haematoxylin were used to stain most of the sections.

### STEM STRUCTURE IN GENERAL

Before one can understand the origin of adventitious roots of purslane, it is necessary to know something about its general morphology. The purslane stem is of a common herbaceous type with discrete bundles. The pri-

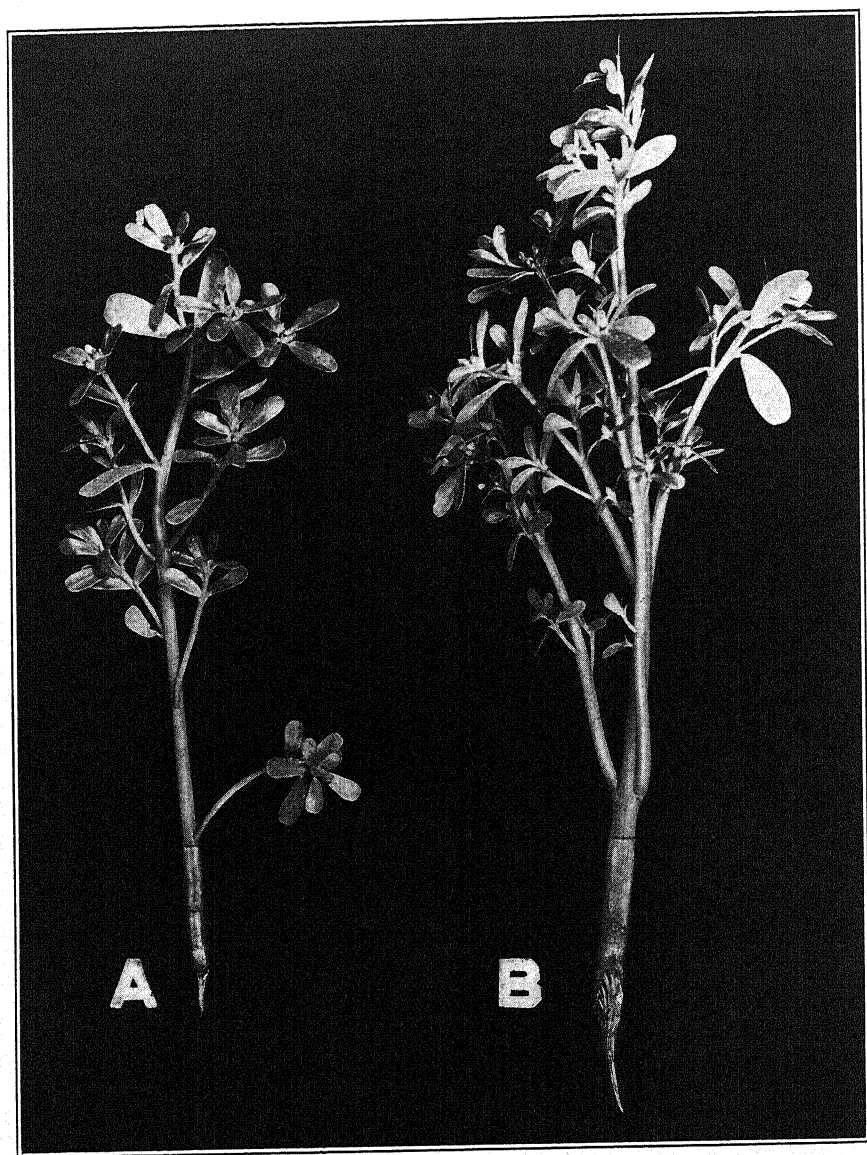


FIGURE 1. Cuttings of *Portulaca oleracea* showing roots emerging from the cut surface. The epidermis had been removed from the basal part of A in an attempt to induce the roots to emerge through the cortex instead of the cut surface.

mary vascular system consists of four or five main bundles which divide, forming traces for leaves and branches. The branch traces are formed a short distance (about 2 mm.) below the point where they leave the stem so that they appear only in sections near a node (Pl. I, 1). On the other hand, as soon as one set of leaf traces leaves the stem the main bundles start branching to form those for the leaf directly above so that the leaf traces are seen throughout the internodes. In any internode there are present the main bundles alternating with groups of from one to three leaf traces making from 10 to 20 bundles in the stem. As the stem grows older some secondary tissues develop both from the fascicular and interfascicular cambiums. The activity of the latter is largely confined to the formation of small bundles (Pl. I, 4) which run more or less obliquely connecting two bundles, generally a main bundle and a leaf trace.

The stem is surrounded by a single, though occasionally double, layer of epidermal cells. Periderm may occur through wounding but is not normally produced. Within the epidermis are two to three layers of collenchyma cells with heavy cellulose walls. There are four to six more layers of cortical cells before reaching the endodermis or starch sheath. These cells are intermediate in size between those of the cortex and the smaller ones of the ray and are somewhat less spherical than either. No thickening of their walls has been observed. Occasionally the cells contain more starch than the surrounding tissue but it is often difficult to distinguish this layer especially when distorted by root growth. Immediately within the endodermis, outside of the vascular bundles, are groups of bast fibers. These and the single layer of cells in the ray in line with them and adjacent to the endodermis have been considered to constitute the pericycle. On its inner side the pericycle throughout most of the ray adjoins the interfascicular cambium.

#### ADVENTITIOUS ROOTS

A single cutting may produce more than a hundred roots but usually the number is nearer fifty. There is no apparent regularity in arrangement nor in order of development. The roots generally arise in the rays adjacent to the primary bundles. In older stems they may also appear adjacent to the smaller connecting bundles or completely independent of any previously formed vascular tissues (Pl. I, 3 and 4). The root primordia are often located one above the other along the vascular bundles (Pl. II, 1). On the average, each bundle has associated with it two or more roots within 2 to 3 mm. of the base of the cutting. More roots are apt to be associated with the larger bundles than with the smaller ones. As many as ten have been observed which had started along one side of a main bundle. Of these the uppermost was no more than 4.5 mm. from the cut surface. When roots arise very close above each other "twin" roots may occur with two steles.



## EXPLANATION: PLATE I

All photomicrographs are  $\times 47$  except 1 and 2, which are  $\times 13$ . Letters designating certain structures have the following meaning: *e*, endodermis; *b*, bast; *p*, pericycle; *c*, cambium.

1. Cross section above the rooting region 2.2 mm. from the base of the cutting. Four of the main bundles have branched, forming branch traces. The other smaller bundles in groups of three are leaf traces.

2. Detail of the upper group of leaf traces of 1.

3. Cross section of a stem 0.4 mm. from the base, showing roots dissolving the cortex.

4. Cross section 0.55 mm. from base, showing the same leaf traces as 2. The roots at the left and right are differentiating. The endodermis has been pushed outward but not dissolved though some other tissue has been dissolved. The group of cells in the center are from a tangential section of a root. Note the activity in the cambium and pericycle and the formation of new vascular elements.

5. Longitudinal section, the lower part of which is about 1 mm. from the base of the cutting. The upper primordium is just inside of the pericycle. Lower primordium shows some division of the pericycle. At the base, tangential divisions of the pericycle form a root still lower.

6. Longitudinal section showing a root differentiated and a space due to partial solution of endodermis.

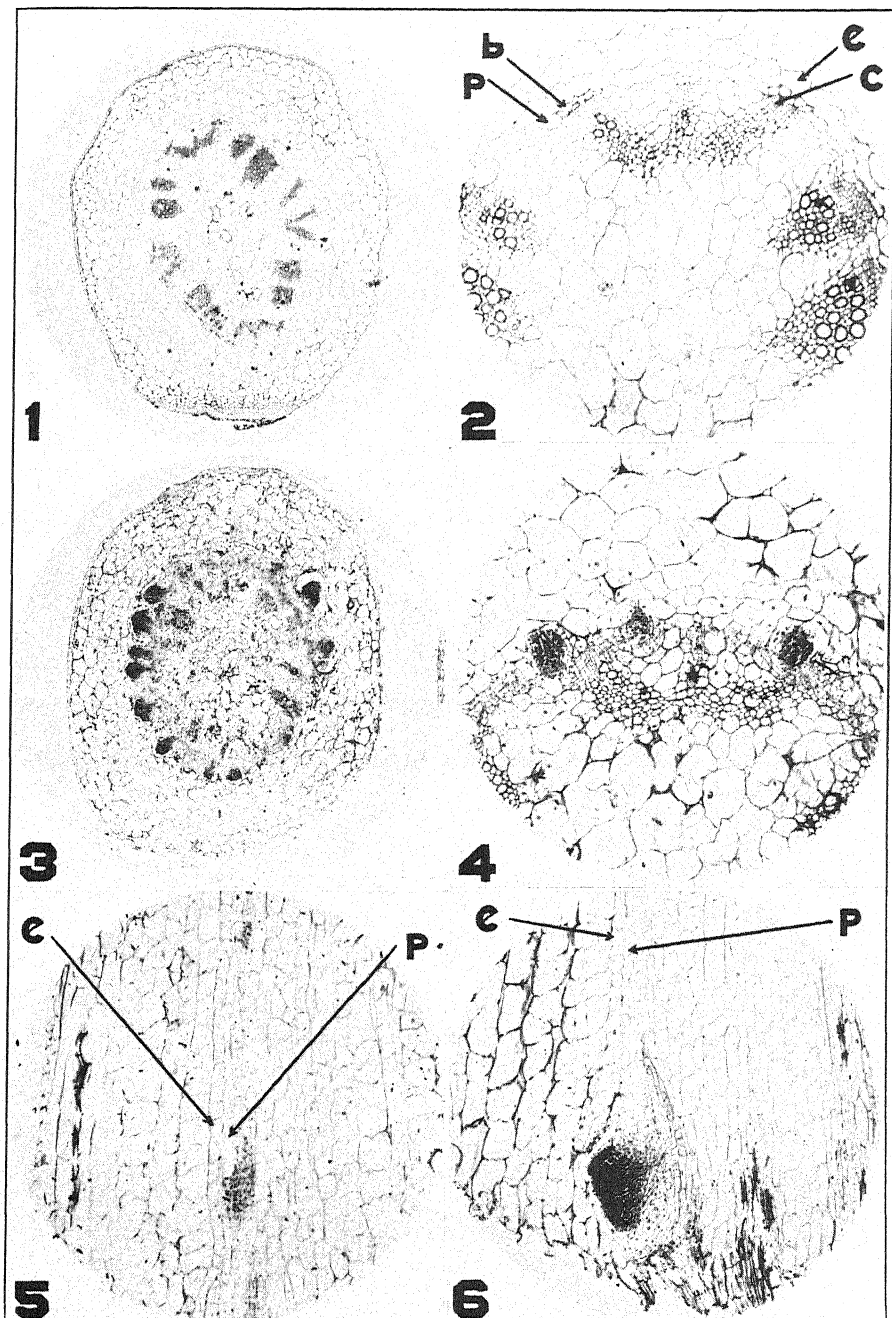


PLATE I—ORIGIN OF ROOTS

## EXPLANATION: PLATE II

All photomicrographs are  $\times 47$ . Letters designating certain structures have the following meaning: *e*, endodermis; *b*, bast; *p*, pericycle; *c*, cambium.

1. Longitudinal section. (Most roots not median.) Five roots, all at about same stage development, have started one above the other.

2. Longitudinal section, showing a mature root which has dissolved a few layers of cortex and turned downward longitudinally to emerge through cut surface.

3. Cross section near base, showing roots growing tangentially. Both are probably inside of the pericycle. In the middle of the cortex is the cross section of an older root which had originated higher up in the stem.

4. Cross section of a stem 0.78 mm. from the base, showing a root starting within pith.

5. Cross section of a stem 0.98 mm. from the base, showing the same bundle as 3. A young root with a digestive pouch is differentiated inside of the bast fibers of the large bundle which is just branching.

6. Cross section 0.88 mm. from the base, showing a root at right well differentiated with pouch. The endodermis is pushed outward but not dissolved. The root at the left is also inside of the endodermis.

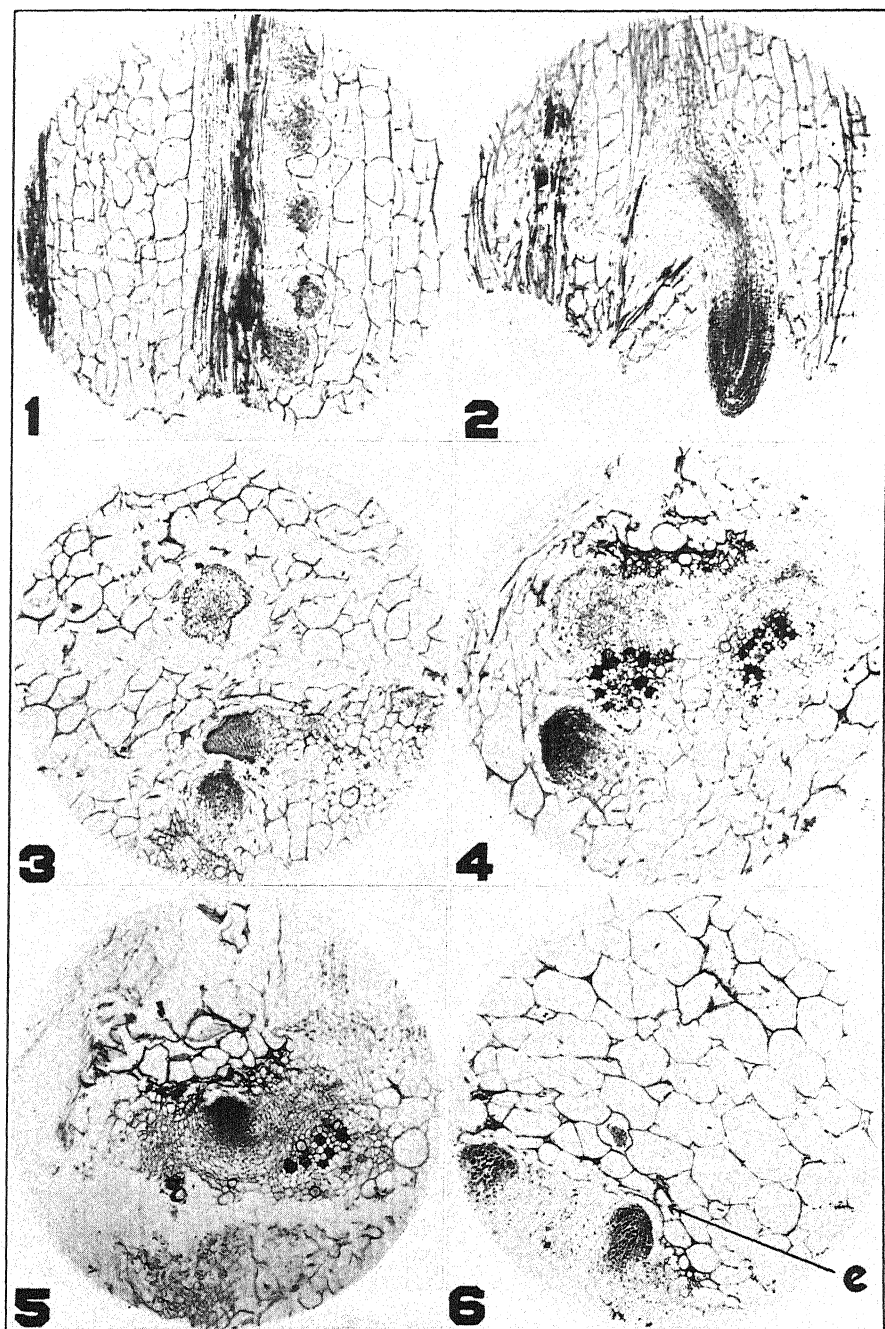


PLATE II—ORIGIN OF ROOTS

The manner of emergence of the roots is peculiar. In most plants adventitious roots grow outward radially from the central cylinder more or less perpendicular to the axis of the stem and emerge through the epidermis or periderm. In purslane the roots emerge through the cut surface and parallel to the main axis of the stem (Fig. 1). Those nearest the base assume this longitudinal direction from the start. The majority of the roots start out radially as in other plants (Pl. I, 3 and Pl. II, 1) but, when about half the cortex has been penetrated, they turn downward and proceed longitudinally (Pl. II, 2 and 3) until they can make their way through the cut surface. When one root is directly above another, the upper goes farther out into the cortex and then turns downward. A crowding of the roots may force the epidermis to split and through this opening roots sometimes emerge. If enough of the stem cortex is removed by a tangential cut a root may come out nearly perpendicular to the axis. When, however, the epidermis with only a small portion of the cortex is removed the roots are not induced to continue their radial growth but turn and go longitudinally as described above (Fig. 1, A).

The factors controlling this direction of growth have not yet been ascertained. The manner of emergence cannot be modified by changing the position of the cutting in relation to gravity. The experiments in which the epidermis was removed as well as the observations on the nature of the outer tissues of the stem seem to preclude the possibility of physical obstruction. Roots penetrate woody tissues or similar herbaceous ones in many plants. Colorimetric determinations of the pH values did not reveal any gradient from the interfascicular cambium to the epidermis. There is, possibly, some chemical condition which favors growth in the direction of the cut surface. In this connection it may be noted that in several instances when the pith of the cutting was decaying at the base, roots grew toward, or actually arose in, the pith (Pl. II, 4).

In the normal purslane stem very little if any cell division takes place in the cambium. In the cutting no callus is formed (Pl. II, 1 and 2) although occasional cell divisions occur in the pith or cortex. All the meristematic activity of the cutting, therefore, is associated with the formation of adventitious roots. The first signs of meristematic activity occur in the cambium two to four days after the cutting is made. In younger stems this activity is usually confined to cells of the interfascicular cambium adjacent to the bundles. The cells divide periclinally and anticlinally. In older stems the activity is not localized at first but divisions take place throughout most of the interfascicular cambium (Pl. I, 4) and often the fascicular cambium as well. In certain regions, however, division is more rapid and continues longer so that soon definite groups of cells are differentiated as in younger stems. Often the ray cells between the cambium and pericycle may take part. In each case the primordia are inside the pericycle, irregu-

lar in shape and size and show no clear differentiation. The cells produced by cambial activity which do not contribute directly to these new roots may form the normal small connecting bundles or connections linking the vascular systems of root and stem (Pl. I, 4). In well rooted cuttings the rays become filled with irregular masses of vascular elements.

In the next stage of root development the pericycle becomes involved in the activity (Pl. I, 5). There are a few tangential and a number of radial divisions. These occur only above the primordia and form the cortex and epidermis of the roots. In Plate I, 4, definitely differentiated roots may be seen just within the endodermis. Differentiation is not always evident even at this period but becomes so as the endodermis is pushed outward and dissolved (Pl. I, 6). That solution rather than crushing occurs seems to be indicated by the fact that the growing roots do not appear to press against the tissue but rather to be separated from it by considerable space.

#### VARIATIONS FROM THE USUAL MODE OF DEVELOPMENT

There are numerous exceptions to the general mode of initiation as outlined above. Not only is the normal activity of the fascicular cambium stimulated at times but root primordia occur within the bundle (Pl. II, 5). The exact tissues involved are not known. Since the bundles often branched just above the point where such a primordium appears the ray parenchyma as well as cambium or phloem may take part in forming these primordia. Since radial growth of these primordia is restricted by the bast fibers, further growth must be in a longitudinal direction. In Plate II, 5, although the bundle has branched, the bast still covers both parts. The root in the narrow ray is well developed and the break between it and the fibers suggests that it has destroyed some of the cells within the bast fibers. In the formation of such roots the pericycle cannot take part. Often also the contribution of the pericycle is dispensed with in roots which from the earliest divisions grow tangentially rather than radially. In these cases the pericycle appears to have been dissolved and even with roots normally oriented, the space between the root and the stem while the endodermis is still untouched may mean solution of at least part of the pericycle (Pl. II, 6). The root would then be formed entirely from cambial derivatives or the pericycle would contribute only part of the cells. In all the examples just mentioned the pericycle does not necessarily make the outer tissues of the root. Likewise there is no evidence that roots originate from the pericycle alone. As mentioned above, roots in two cases were found to have been formed entirely within the pith (Pl. II, 4) and unconnected with the cambium. With this exception the activity of the interfascicular cambium appeared to be the most essential feature of the formation of the adventitious roots of purslane.

## SUMMARY

1. The majority of adventitious roots from cuttings of *Portulaca oleracea* L. arise in the medullary rays within 5 mm. of the base, and emerge through the cut surface without penetrating the epidermis.

2. The earliest meristematic activity is in the interfascicular cambium. Cells derived from these divisions form the inner portions of the root and accessory vascular tissues of the stem.

3. The pericycle generally forms the outer portions of the root, the endodermis being the first tissue dissolved by the root.

4. Instances occur in which the roots are fully differentiated and some tissues already dissolved with the endodermis still intact. Some primordia were found within vascular bundles where the pericycle could not take part in their formation.

5. Under some conditions roots arise within the pith.

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## A CAPILLARY GLASS ELECTRODE

W. J. YODEN AND I. D. DOBROSKY

### INTRODUCTION

The advent of the quinhydrone electrode greatly facilitated the measurement of hydrogen ion concentration in many types of solutions. It eliminated the bulky source of hydrogen gas, the difficulties with solutions which foamed excessively, and particularly the troublesome preparation of electrodes which frequently became poisoned and useless. Especially gratifying was the greatly diminished time required for equilibrium. It was not all clear gain, however, and a new crop of disadvantages arose with the new electrode. The quinhydrone electrode finds its special province in acid solutions, and in practice does not give accurate values in solutions more alkaline than pH 8. Especially is this so in slightly buffered solutions. Again, questions are frequently raised as to the possible interaction of the added quinhydrone with material in solution. The performance of measurements on very small samples was difficult since tests demonstrated that it was not desirable to diminish the electrode area.

The glass electrode is now on trial and is not without its list of objections. The voltage-measuring devices used with the hydrogen electrode required no modification for work with the quinhydrone electrode. Most of them will not serve for use with the glass electrode. This is no criticism of the glass electrode save that the arrangements frequently proposed appear to be vastly more intricate than the familiar devices. This is not necessarily so. More pertinent was the difficulty in preparing the electrodes and their extreme fragility. These points are fairly eliminated in the device described in this paper and in addition the quantity of solution required has been reduced below one-hundredth of a cubic centimeter.

In the brief span of years since Kerridge's paper (8) on the use of the glass electrode in biochemistry, many workers have published on the subject. It appears that more attention has been devoted to the problem of designing a voltage-measuring device than to developing a satisfactory form of the glass electrode. Four methods of measuring the voltage have been advanced. Early workers used sensitive quadrant electrometers as a current detecting device. Kerridge (8) used the Dolezalek electrometer and stated that the Lindemann was not sensitive enough but in a later paper (9) used the Lindemann. Several papers by Partridge (14), Elder and Wright (2), Harrison (5), Fosbinder and Schoonover (3), Stadie (17), Greville and MacLagan (4) are largely concerned with describing electrical arrangements which depend for their sensitivity on vacuum tubes, such as are used in radio receiving sets. So persistently is this general method advanced that the senior author, after the apparatus described in



this paper was complete and in daily use, built a voltage measuring device of this variety. Although there was available a very recent product (Pilotron FP-54) in the art of tube manufacture, indeed a tube especially designed for measuring very minute currents of the order of  $10^{-14}$  amperes, it was early apparent that the equipment required more room, was very much heavier, and involved storage batteries and their frequent recharging. The construction in the laboratory of this equipment was also more difficult. In addition, there is no saving in cost as the tube and the required sensitive galvanometer and accessories at least equalled the outlay for an electrometer.

Morton (13) employed a ballistic galvanometer. An undesirable feature of this method is the need to time accurately with a stop watch the period during which the condenser is charged by the electrode system. The method also calls for the use of many buffers of known pH. An attempt has been made by Robertson (16) to decrease the resistance of the electrode system sufficiently to permit measurements of limited accuracy with an ordinary galvanometer.

Most of the electrodes described are of bulb shape or bulbs with depressions to hold the sample. MacInnes and Dole (10, 11) describe a form which permits the use of very thin films (one micron) of glass and consequently attains a low resistance. It is capable of using as little as two drops as a sample. The preparation of this form involves considerably more skill than the capillary type described in this paper.

#### THE NATURE OF THE GLASS ELECTRODE

If a very thin glass wall separates two aqueous solutions there exists a difference of potential between the fluids which in general bears a simple relation to their difference in pH. The character of this relationship may be readily illustrated. Let us take a thin-walled soft glass bulb filled with a buffer solution whose pH need not be known. Immerse the bulb in a convenient solution A and determine the potential. Choose a second solution B and obtain the potential between it and the liquid in the bulb. Now subject these two liquids to measurements with the hydrogen and quinhydrone electrodes. The results of an experimental trial are given below:

	Glass Electrode	Hydrogen Electrode	Quinhydrone Electrode
Solution A	117 mv.	481 mv.	218 mv.
Solution B	<u>47 mv.</u>	<u>551 mv.</u>	<u>148 mv.</u>
	70 mv.	70 mv.	70 mv.

That is, when the different sets of potentials obtained are compared there is a common difference between them. At 25° C. the difference in pH between solutions A and B is obtained by dividing 70 by 59.1. At other

temperatures the factors for converting millivolts to pH may be found in column 3, Table C in the appendix of Clark's textbook on "The determination of hydrogen ions" (1). The values given in the table must be multiplied by 1,000 since the table is based on volts instead of millivolts. The absolute values for the e.m.f. in the above cases depend on the liquid within the bulb for the glass electrode and on the type of reference half cell in the other cases. The values for the hydrogen and quinhydrone electrodes were obtained with a saturated calomel half cell; the usual formulas for pH then give 3.98 and 5.16 respectively for A and B. If the pH of either liquid is known the pH of the other may be obtained using the glass electrode data.

#### DESCRIPTION OF THE ELECTRICAL APPARATUS

Although the glass electrode, like the hydrogen and quinhydrone electrodes, is an electrometric method, the apparatus usually employed for electromotive force measurements is not suitable. In general the principle underlying voltage measurement is that of the potentiometer. The potentiometer offers a means of obtaining a known and variable e.m.f. which is connected in opposition to the unknown potential with a galvanometer or current-detecting instrument in one of the leads. The known voltage is varied until the galvanometer shows that no current flows between the systems. It then follows that the known and unknown voltages are equal. A tapping key insures a minimum of current drain on the experimental cell during the measurement. The immediate difficulty encountered with the glass electrode is that the thin wall of glass separating the liquids offers such a high resistance to the passage of current that even a very sensitive galvanometer is not equal to the task of indicating the inequality of the opposed voltages. Cases of this kind fall within the province of the quadrant electrometer. Most designs of this instrument call for refined technique in operating. The Lindemann electrometer, however, is rugged, very compact, and requires no leveling. Its moving system is so small that the entire instrument is placed on a microscope stage and the needle observed through an eyepiece with a micrometer scale. The high sensitivity of this instrument necessitates shielding the system from stray electrical charges. Especial efforts must also be taken throughout in the matter of insulation. These considerations will serve to show the purpose back of the features of the apparatus.

The entire assembly is contained in a box (Fig. 1) about 30 cm. wide, 30 cm. high, and 37.5 cm. deep. The box is constructed of metal (or wood lined with metal) and serves as shield and ground. The circuits to be described are arranged on panels on either side of the box. The rheostat knobs and push buttons extend through to the outside. In the back of the box are two 22 volt "C" batteries, one 7.5 volt "C" battery, and a 1.5 volt

dry cell. The front of the box provides space for the electrode assembly and the microscope. An opening in the top provides for the projecting eyepiece of the microscope and a window in the side for illumination. Accessibility

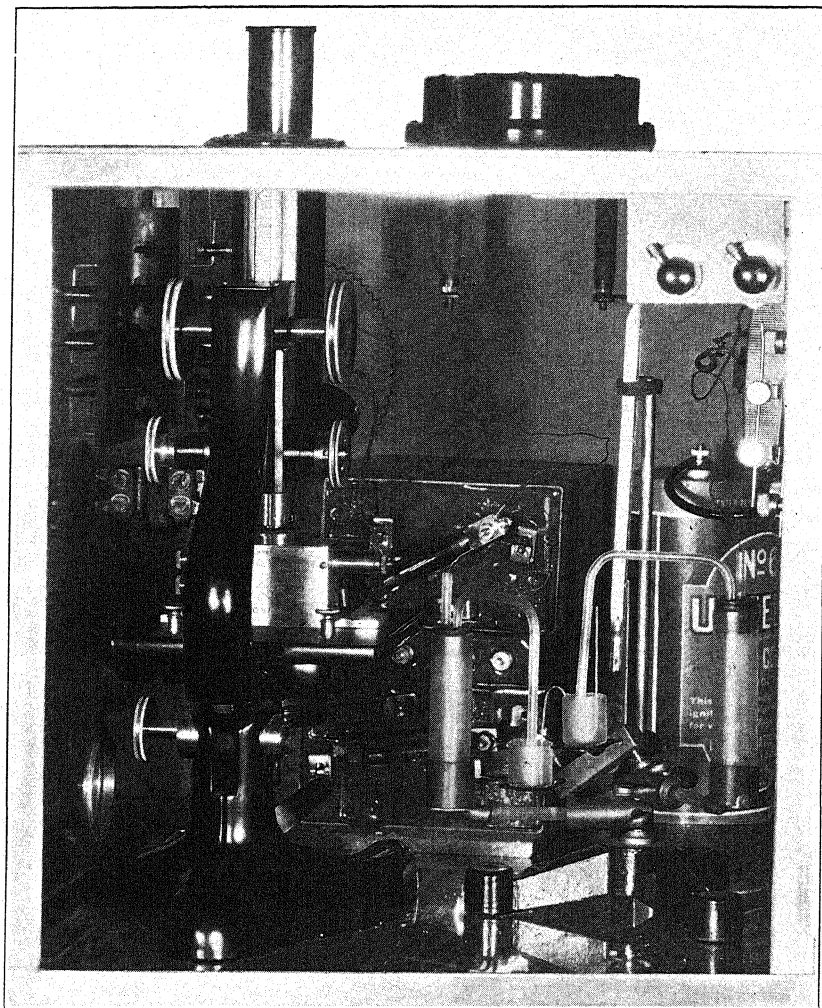


FIGURE 1. Interior view of metal box which houses complete equipment for determinations of pH with the glass electrode.

to all parts without disturbing the assembly is attained by making the top of the box removable. The millivoltmeter is mounted on this removable top.

There are three electrical circuits involved (Fig. 2). A known variable voltage is set up by the dry cell acting through a fixed ten ohm resistance in series with a six ohm rheostat across which a millivoltmeter (300 mv. scale) is connected. In another circuit one pair of the electrometer quadrants is brought to approximately 22 volts above ground and the other about 22 volts below ground by means of the "C" batteries. Grid leaks are mounted on the batteries and prevent damage in case of accidental contact. A double-pole double-throw switch is provided for grounding the quadrants or connecting them to the batteries. The 7.5 volt "C" battery mentioned above is not shown in Fig. 2. One or more units of this battery are included in series with the 22 volt blocks to permit altering the voltage on the quadrants by reasonably small units. It was also found convenient

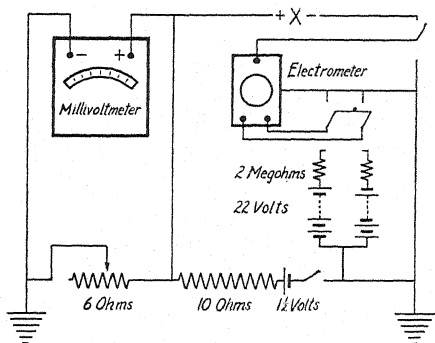


FIGURE 2. Diagram of the electrical circuits.

to connect a 1,000 ohm rheostat of the potentiometer type across one of the units and connect the contact arm of the rheostat to the ground in place of the ground connection shown. In this way the relative voltages imposed on the two sets of quadrants could be varied practically continuously. The third circuit involves the electrometer needle. The needle is connected to a single-pole double-throw switch so that it may be connected first directly to the ground and then to the ground through the electrode system, X, in series with and opposing the known voltage indicated by the millivoltmeter. If these two voltages are equal and opposite, the position of the needle remains unaltered from that taken up when connected directly to the ground. Both double-throw switches are of especial construction. For the single-pole switch a binding post is sealed with sealing wax in a short piece of quartz tubing and this in turn mounted with sealing wax in the panel. The needle of the electrometer is connected to this binding post which also carries a piece of spring nickel about three inches long. Another binding post mounted similarly an inch and a half from the first carries a wire that connects with the electrode system. The

spring normally presses against this post, thus connecting the needle with the electrode system. Near the end of the piece of spring a piece of quartz tubing mounted in the panel carries a quartz rod that rests on the spring and projects through the side of the box. By depressing this rod the spring is bent inward breaking contact with the second binding post and making contact with a piece of bus bar which is grounded. The other switch is built up in the same way save that one quartz rod with a horizontal bar serves to depress both pieces of spring and ground them. The electrometer case is also permanently grounded. To determine the voltage of an unknown system with the apparatus, one closes the switch that is connected to the dry cell. The millivoltmeter then shows a reading which may be varied by adjusting the six ohm rheostat. The double-pole switch is momentarily thrown to the ground and then released so that the quadrants are left connected to the 22 volt "C" batteries. The needle of the electrometer is then grounded by depressing and holding down the quartz rod which controls the switch. While the needle is grounded, and hence at zero potential, the position of the needle image on the scale in the eyepiece of the microscope is noted. On releasing the rod the needle is automatically connected with the unknown system which, in turn, is in series with the known voltage and the ground. If the unknown voltage does not cancel the known voltage the net unbalanced voltage causes the electrometer needle to take up some other position. The six ohm rheostat is then adjusted until the image coincides with the position taken up when the needle is directly grounded. When this is accomplished the rod is depressed to check the result and any slight further adjustment made after releasing the rod. The voltage of the unknown system is equal to that shown on the millivoltmeter scale. The sensitivity of the electrometer is such that the voltage may be easily adjusted to the half millivolt. Using a 16 mm. objective and a 15x ocular, a sensitivity of 10 eyepiece divisions for an e.m.f. of 30 or 40 millivolts is possible. The sensitivity of the electrometer increases as the voltages imposed on the quadrants of the electrometer approach the critical values furnished by the maker of the instrument. In the apparatus described the voltage readings were made on a small millivoltmeter whose scale was subdivided in five millivolt divisions.

#### DESCRIPTION OF GLASS ELECTRODES USED

*Bulb.* Many measurements were made with a bulb blown from a piece of ordinary soft glass tubing previously drawn out to a capillary. The bulb, platform for supporting the cup of external liquid, and the calomel half cells are shown in Figure 3. Short rods of quartz support all four items and provide insulation. Ordinary battery connectors with a hole drilled through one end perpendicular to the long axis of the connector form a simple means of clamping the quartz rods to the stand. A modified bulb with a depres-

sion for holding the external liquid was also used for small quantities. This was abandoned in favor of the new type.

*Capillary.* A thin-walled soft glass test tube was used to prepare capillaries not over 0.5 mm. in external diameter. Measurements on a number of these capillaries show variation in external diameter between the limits 0.40 mm. and 0.50 mm. The thickness of the wall of the capillary averaged about 20  $\mu$ . The ratio of wall thickness to capillary diameter was sub-

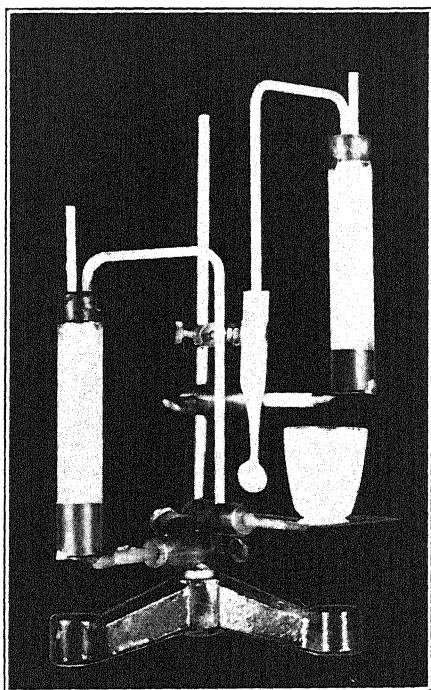


FIGURE 3. Supporting stand with bulb type of glass electrode, cup, and calomel half cells.

stantially that of the original test tube. The volume of these capillary electrodes was checked in several cases by filling with water and weighing. The weight of water was often less than 10 milligrams corresponding to a sample of 0.01 cc. or less. The electrical resistance of the electrodes is several hundred megohms and may be as much as 1,000 megohms. The use of special glasses (12) that have been found particularly adapted for preparing other types of glass electrodes would diminish these values. Inasmuch as satisfactory measurements were obtained with capillaries drawn from soft glass test tubes, the employment of special glass is a matter of choice.

A piece of capillary a few centimeters long was bent into an exaggerated S (Fig. 4). No difficulty arises in making these bends if a small electric heater is used to soften the glass. A very simple one may be constructed by winding a few turns of nichrome wire on a piece of quartz rod and setting this across the top of a Gooch crucible bringing the leads through the holes in the bottom. A piece of mica over the top of the crucible provides a flat surface for the capillary to rest on while it is shaped. If sharp bends are avoided the capillary is practically immune from breakage. These capillaries readily fill themselves when brought in contact with the sample. This makes possible the collection of a fraction of a drop at the source of the sample and entirely avoids any manipulation of the fluid. The capillary is



FIGURE 4. View of capillary glass electrode and the method of rinsing the electrode.

then placed (Fig. 5) so that one of the bends is immersed in a small cup of the reference fluid with the tip extending into a cup of saturated potassium chloride solution. In this way the sample is completely set apart from the external liquid by the thin walls of the capillary and contact also established with the liquid in the capillary. The side arms of two calomel half cells dip into the potassium chloride solution and into the reference solution and are not disturbed during a series of measurements.

#### METHOD OF MAKING A DETERMINATION

The procedure followed for a pH determination requires that the capillary be first filled with a solution of known acidity and the resulting e.m.f. determined. A convenient solution for this purpose is a  $M/20$  solution of potassium acid phthalate. The electrode is then removed, washed exter-

nally and internally, dried, and filled with the unknown. The e.m.f. is again determined and the difference found between this value and the first e.m.f. obtained. This difference in millivolts divided by the factor 59.1 (for 25° C.) gives the difference in pH between the phthalate solution and the unknown. If the reference solution which bathes the capillary is a neutral buffer the voltage obtained with the phthalate in the capillary is in the neighborhood of 180 mv. Solutions more alkaline than the phthalate give values less than this, more acid solutions give larger readings.

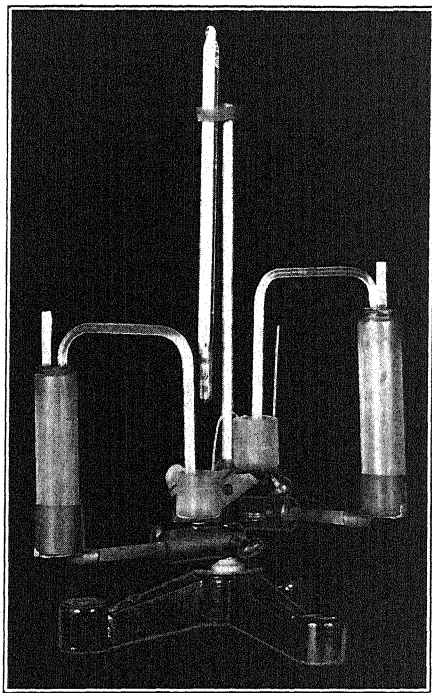


FIGURE 5. Electrode assembly showing the capillary glass electrode, cups, and calomel half cells.

The difference in millivolts, after converting to pH by dividing by the factor 59.1 (when measurements are made at 25° C.), is added to or subtracted from 3.98 (the pH of the phthalate solution), depending on whether the voltage obtained with the unknown is smaller or larger than the voltage set up by the phthalate solution. It follows that the voltage becomes smaller as the unknown becomes more alkaline. Near the neutral point the observed voltage is zero and beyond this the voltage is in the opposite direction, requiring that the wires leading to the calomels be interchanged to



effect a measurement. A voltage so obtained is marked with a negative sign and hence when the difference between it and the phthalate value is computed the two values are added. Table I has been arranged for converting the difference in millivolts between the voltage obtained with a M/20 potassium acid phthalate solution and the voltage with the un-

TABLE I  
PART I\*  
FOR USE IN CONVERTING MILLIVOLTS TO pH AT 25° C.

E (mv.)	0	1	2	3	4	5	6	7	8	9
0	3.98	3.99	4.01	4.03	4.05	4.06	4.08	4.10	4.11	4.13
1	4.15	4.16	4.18	4.20	4.21	4.23	4.25	4.27	4.28	4.30
2	4.32	4.33	4.35	4.37	4.38	4.40	4.42	4.43	4.45	4.47
3	4.49	4.50	4.52	4.54	4.55	4.57	4.59	4.60	4.62	4.64
4	4.65	4.67	4.69	4.71	4.72	4.74	4.76	4.77	4.79	4.81
5	4.82	4.84	4.86	4.87	4.89	4.91	4.93	4.94	4.96	4.98
6	4.99	5.01	5.03	5.04	5.06	5.08	5.09	5.11	5.13	5.15
7	5.16	5.18	5.20	5.21	5.23	5.25	5.26	5.28	5.30	5.31
8	5.33	5.35	5.37	5.38	5.40	5.42	5.43	5.45	5.47	5.48
9	5.50	5.52	5.53	5.55	5.57	5.59	5.60	5.62	5.64	5.65
10	5.67	5.69	5.70	5.72	5.74	5.75	5.77	5.79	5.81	5.82
11	5.84	5.86	5.87	5.89	5.91	5.92	5.94	5.96	5.97	5.99
12	6.01	6.03	6.04	6.06	6.08	6.09	6.11	6.13	6.14	6.16
13	6.18	6.19	6.21	6.23	6.24	6.26	6.28	6.30	6.31	6.33
14	6.35	6.36	6.38	6.40	6.41	6.43	6.45	6.46	6.48	6.50
15	6.52	6.53	6.55	6.57	6.58	6.60	6.62	6.63	6.65	6.67
16	6.68	6.70	6.72	6.74	6.75	6.77	6.79	6.80	6.82	6.84
17	6.85	6.87	6.89	6.90	6.92	6.94	6.96	6.97	6.99	7.01
18	7.02	7.04	7.06	7.07	7.09	7.11	7.12	7.14	7.16	7.18
19	7.19	7.21	7.23	7.24	7.26	7.28	7.29	7.31	7.33	7.34
20	7.36	7.38	7.40	7.41	7.43	7.45	7.46	7.48	7.50	7.51
21	7.53	7.55	7.56	7.58	7.60	7.62	7.63	7.65	7.67	7.68
22	7.70	7.72	7.73	7.75	7.77	7.78	7.80	7.82	7.84	7.85
23	7.87	7.89	7.90	7.92	7.94	7.95	7.97	7.99	8.00	8.02
24	8.04	8.06	8.07	8.09	8.11	8.12	8.14	8.16	8.17	8.19
25	8.21	8.22	8.24	8.26	8.28	8.29	8.31	8.33	8.34	8.36
26	8.38	8.39	8.41	8.43	8.44	8.46	8.48	8.50	8.51	8.53
27	8.55	8.56	8.58	8.60	8.61	8.63	8.65	8.66	8.68	8.70
28	8.71	8.73	8.75	8.77	8.78	8.80	8.82	8.83	8.85	8.87
29	8.88	8.90	8.92	8.93	8.95	8.97	8.99	9.00	9.02	9.04
30	9.05	9.07	9.09	9.10	9.12	9.14	9.15	9.17	9.19	9.21
31	9.22	9.24	9.26	9.27	9.29	9.31	9.32	9.34	9.36	9.37
32	9.39	9.41	9.43	9.44	9.46	9.48	9.49	9.51	9.53	9.54
33	9.56	9.58	9.59	9.61	9.63	9.65	9.66	9.68	9.70	9.71
34	9.73	9.75	9.76	9.78	9.80	9.81	9.83	9.85	9.87	9.89
35	9.90	9.92	9.93	9.95	9.97	9.98	10.00	10.02	10.03	10.05
36	10.07	10.09	10.10	10.12	10.14	10.15	10.17	10.19	10.20	10.22
37	10.24	10.25	10.27	10.29	10.31	10.32	10.34	10.36	10.37	10.39
38	10.41	10.42	10.44	10.46	10.47	10.49	10.51	10.53	10.54	10.56
39	10.58	10.59	10.61	10.63	10.64	10.66	10.68	10.69	10.71	10.73

\* See footnote under Part 2 of this table.

known solution, directly into the pH of the unknown. It is divided into two parts. Part 1 is entered when the difference is the result of voltage smaller than the phthalate value; Part 2 when the difference results from a larger e.m.f. than the phthalate value. The table is independent of the pH of the reference buffer which is in contact with the outside of the capillary.

TABLE I  
PART 2\*  
FOR USE IN CONVERTING MILLIVOLTS TO pH AT 25°C.

E (mv.)	0	1	2	3	4	5	6	7	8	9
0	3.98	3.96	3.94	3.93	3.91	3.89	3.88	3.86	3.84	3.83
1	3.81	3.79	3.77	3.76	3.74	3.72	3.71	3.69	3.67	3.66
2	3.64	3.62	3.61	3.59	3.57	3.56	3.54	3.52	3.50	3.49
3	3.47	3.45	3.44	3.42	3.40	3.39	3.37	3.35	3.34	3.32
4	3.30	3.28	3.27	3.25	3.23	3.22	3.20	3.18	3.17	3.15
5	3.13	3.12	3.10	3.08	3.06	3.05	3.03	3.01	3.00	2.98
6	2.96	2.95	2.93	2.91	2.90	2.88	2.86	2.84	2.83	2.81
7	2.79	2.78	2.76	2.74	2.73	2.71	2.69	2.68	2.66	2.64
8	2.62	2.61	2.59	2.57	2.56	2.54	2.52	2.51	2.49	2.47
9	2.46	2.44	2.42	2.40	2.39	2.37	2.35	2.34	2.32	2.30
10	2.29	2.27	2.25	2.24	2.22	2.20	2.18	2.17	2.15	2.13
11	2.12	2.10	2.08	2.07	2.05	2.03	2.02	2.00	1.98	1.96
12	1.95	1.93	1.91	1.90	1.88	1.86	1.85	1.83	1.81	1.80
13	1.78	1.76	1.74	1.73	1.71	1.69	1.68	1.66	1.64	1.63
14	1.61	1.59	1.58	1.56	1.54	1.53	1.51	1.49	1.47	1.46
15	1.44	1.42	1.41	1.39	1.37	1.36	1.34	1.32	1.31	1.29
16	1.27	1.25	1.24	1.22	1.20	1.19	1.17	1.15	1.14	1.12
17	1.10	1.09	1.07	1.05	1.03	1.02	1.00	.98	.97	.95
18	.93	.92	.90	.88	.87	.85	.83	.81	.80	.78
19	.76	.75	.73	.71	.70	.68	.66	.65	.63	.61
20	.59	.58	.56	.54	.53	.51	.49	.48	.46	.44
21	.43	.41	.39	.37	.36	.34	.32	.31	.29	.27
22	.26	.24	.22	.21	.19	.17	.15	.14	.12	.10
23	.09	.07	.05	.04	.02	.00				

\* The use of this table requires that M/20 potassium acid phthalate be used as a reference standard for comparison. Part 1 is used when the voltage obtained with the unknown solution is smaller than the voltage found with the phthalate. The table is entered with the difference of the readings. Part 2 is used when the voltage with the unknown is larger than the voltage for the phthalate. The difference between the values is used in entering the table.

The purpose of using an intermediary solution instead of comparing the unknown and phthalate solutions directly by placing the phthalate solution in the cup is the elimination of a number of sources of error. It is essentially a method of substitution and in the process of taking differences any constant errors which appear in the observed voltages are automatically cancelled. Such errors are an inaccurate zero setting of the voltmeter, inequality of the two calomel half cells, and liquid junction potentials at the calomel half cells.

The success of the capillary electrode depends to a considerable degree on finding some means of washing it. In the method developed about two or three cubic centimeters of distilled water are forced rapidly through the capillary. This is really equivalent to several hundred rinsings. The capillary is taken between the thumb and forefinger with the tip barely protruding. The opening of a medicine dropper is placed over the tip (Fig. 4), the thumb and forefinger brought together to form a seal, and the bulb compressed. At first there is some tendency to break off small portions of the

TABLE II  
A COMPARISON OF THE PH VALUES OF FRUIT JUICES BY DIFFERENT ELECTRODES

Material	Glass	Quin- hydrone	Hydro- gen	Sample from hydrogen electrode determination	
				Glass	Quin- hydrone
Peach ( <i>Prunus persica</i> (L.) Stokes)	3.55	3.49	3.50	3.58	3.49
Apple ( <i>Pyrus malus</i> L.)	3.41	3.49	3.49	3.50	3.50
" " " " "	3.65	3.57			
" dil. 1:5	3.72	3.71	3.68	3.80	3.72
" rotten	3.36	3.23	3.28	3.38	3.24
Grape ( <i>Vitis labrusca</i> L.)	3.48	3.46	3.51	3.53	3.46
" dil. 1:15	3.62	3.65	3.65	3.62	3.65
Corn ( <i>Zea mays</i> L.)	6.14	6.02	6.07	6.10	6.04
<i>Ilex verticillata</i> Gray	5.24	5.29	5.31	5.26	5.34
<i>Photinia villosa</i> D. C.	5.31	5.24	5.26	5.33	5.31
<i>Cotoneaster simonsii</i> Baker	4.72	4.65	4.70	4.70	4.66
<i>Viburnum acerifolium</i> L.	5.11	5.16	5.12		
" <i>americanum</i> Auth.	2.86	2.93	2.94	2.96	2.94
" <i>dilatatum</i> Thunb.	2.76	3.00	2.70	2.77	3.00
" " dil. 1:5	2.81	2.85	2.84	2.83	2.86
" <i>opulus</i> L.	2.86	2.81	2.79	2.86	
" " dil. 1:5	2.94	2.89	2.93	3.00	2.91
" " " 1:25	3.20	3.09	3.19	3.18	3.11
Tomato red ( <i>Solanum lycopersicum</i> L.)	3.99	4.07	3.98	3.97	4.10
" green	3.99	3.97	3.92	3.90	3.97
" frozen	4.28	4.26	4.27	4.28	4.27
" " dil. 1:5	4.41	4.38	4.36	4.43	4.38
" green	4.43	4.33	4.36	4.45	4.35
" " filt. + tol.	4.32	4.35	4.29	4.32	4.36
5 green tomatoes filt. + tol.	4.26	4.24	4.28	4.31	4.24
5 " " " dil. 1:10	4.43	4.41	4.45	4.48	4.41
5 red tomatoes filt. + tol.	4.24	4.20	4.22	4.30	4.20
5 " " " dil. 1:10	4.33	4.31	4.33	4.33	4.34
5 red rotten tomatoes filt. + tol.	4.63	4.51	4.48	4.62	4.51
5 " " " " dil. 1:10	4.62	4.65	4.60	4.63	4.67

capillary but with practice this can be entirely avoided. Electrodes have been in use for months so that there is no necessary breakage from the washing. Sometimes in spite of this thorough washing the capillary is not clean enough to pick up the sample. Alcohol or other solvents may be employed in the dropper. More effective than these is warm cleaning solution. The electrode is filled by immersing it in a small beaker of the cleaning mixture and, after two or three minutes, washing it several times with dis-

tilled water. When not in use the electrodes are left in small vials of distilled water or dilute hydrochloric acid. Even if the electrode is set aside for only a minute or two it is a safe precaution to drop it in a vial, as otherwise it may very easily be lost. The electrode is dried with a jet of compressed air.

#### RESULTS OBTAINED WITH THE GLASS ELECTRODE

The main object of the work was the construction of a compact and rugged device employing the glass electrode rather than an investigation of the nature of the glass electrode and its limitations. On one occasion the

TABLE III  
COMPARISON OF PH VALUES OBTAINED WITH DIFFERENT ELECTRODES

Material	Glass	Hydrogen	Quinhydrone
Buttermilk	4.38	4.29	4.34
Fermillac	4.31	4.31	4.34
Milk	6.61	6.58	6.58
Muck soil	5.33		5.41
Muck soil	5.07		5.12
Soil	6.99	6.88	
Sulphite liquor	2.35		
Calcium malate	5.86	5.88	
Boric acid & Ca (OH) <sub>2</sub>	9.88	10.06	
Buffer (Clark & Lubs)	3.25	3.25	
" " "	4.44	4.48	
" " "	5.47	5.51	
" " "	6.84	6.80	
" " "	7.50	7.49	
" " "	7.87	8.18	
Potato juice ( <i>Solanum tuberosum</i> L.)	6.08		6.11
Potato juice & Ca(OH) <sub>2</sub>	7.80	7.74	7.76
" " "	8.62	8.58	
Potato juice & KOH	7.86	7.80	
" " "	8.74	8.81	
<i>Agaricus campestris</i> L.	6.33	6.25	6.31
Black walnut ( <i>Juglans nigra</i> L.)	4.45	4.34	
" " " " dil. 1:10	4.53	4.49	5.04
" " " " dil. 1:100	4.80	4.68	4.82
Green tomato	4.43	4.38	
" " "	4.24	4.20	
" " "	4.21	4.23	
" " "	4.24	4.21	
" " "	4.24	4.23	
Red "	4.24	4.22	
" " "	4.18	4.22	
" " "	4.31	4.38	
" " "	4.14	4.16	
" " "	4.40	4.41	

set was transported several hundred miles on the floor of an automobile and measurements made within an hour at the destination. It seems worth while at this time, however, to include a considerable number of measurements made on biological materials. These determinations are tabulated in Tables II and III. The measurements were made in a routine manner

without attempting to obtain maximum precision. The values obtained with the glass electrode were compared with parallel measurements made as nearly simultaneously as possible with the hydrogen and quinhydrone electrodes. The voltage readings in all methods were recorded only to the nearest millivolt, which corresponds to slightly less than 0.02 pH unit. In the great bulk of acidity determinations in biological work the question of sample variation and other uncontrollable factors make further refinement of the voltage measurement unnecessary.

In the first part of the work it was customary to take three portions of the sample and obtain a reading by each method. The values so obtained are listed in columns 2, 3, and 4 of Table II. When this was done the liquid in the hydrogen electrode vessel was divided and again measured by the glass electrode and the quinhydrone electrode. These determinations are entered in columns 5 and 6 of Table II. It was believed that any alteration in the pH due to the stream of hydrogen would then be detected and that the second set of measurements should show better agreement with the value obtained with the hydrogen electrode. The data show that rarely did the second pair of measurements differ significantly from the first values recorded. In Table III additional determinations with the glass electrode are compared with parallel determinations with the other electrodes. The data show further that whenever discrepancies appeared, diluting the material somewhat brought the various methods into better agreement. The average difference of the glass electrode reading from the mean of the hydrogen and quinhydrone values is less than 0.05 pH. Since the average difference between the values obtained with the hydrogen and quinhydrone electrodes is also about 0.05 pH it would appear that the glass electrode measurements may be fully relied upon. Actually the case for the glass electrode is better than this. In some instances noticeable differences are apparent between the hydrogen and quinhydrone electrodes and the glass electrode may check one or the other of the values so that it is hardly fair to compare the glass electrode reading with the mean of the two. In some cases, as for example muck soil, it was impossible to obtain a hydrogen electrode reading while very fair agreement was found between the glass and quinhydrone electrodes.

#### DISCUSSION

Several studies have demonstrated the quantitative nature of the potential-pH relationship established by the glass electrode. MacInnes and Dole (12) investigated several different glasses in order to determine the most favorable composition. They find a quantitative relationship holds up to a pH of 9.5, although in the more alkaline solutions high concentrations of sodium ion are undesirable. Hill (7) worked with solutions as alkaline as pH 11.4. The MacInnes and Dole form of electrode has been

used by several workers. In this connection Harrison (5) mentioned difficulties in constructing the MacInnes and Dole electrodes. Greville and MacLagan (4) found the same electrodes fragile and also report that a considerable number of those prepared were failures due to small openings that were only detected when put into use. Among the special applications there is the work of Vroegtlin and others (18) on circulating blood, that of Hatos and Goll (6) on soils, and Rabinowitsch and Kargin (15) on colloidal solutions.

The measurement of the potential developed across the membrane has generally been effected either through the use of quadrant electrometers or vacuum tube devices. Elder and Wright (2) maintain that the use of the electron tube enables measurements to be made on humid summer days which otherwise are impossible. The small drying chamber which is an integral part of the Lindemann type of electrometer answers this objection. It is a great advantage to be relieved of the necessity of frequent charging or replacement of batteries. The only battery replaced after a year's use with the apparatus described in this paper was the 1.5 volt dry cell. The vacuum tube arrangement built here and several published illustrations of tube outfits offer a marked contrast to the compact box (Fig. 1) which easily houses electrode system, electrometer and microscope, and all the batteries.

#### SUMMARY

A new type of electrode has been described which offers the advantages of ease of construction, ruggedness, and the employment of extremely small samples. The volume of liquid used has been as little as 0.007 cc. The method of substitution employed eliminates many constant errors and minimizes greatly differences between individual electrodes. The capillary electrode is also excellent for rapidly collecting a sample and thus avoiding exposure to the air either before or during the measurement.

A voltage-measuring device for use with this electrode is also described. Due to the inclusion of the microscope within the metal box the difficult problem of shielding the connecting wires has been easily solved. The arrangement of the electrical circuits is such that all controls and push buttons are at ground potential when touched. This feature adds considerably to the stability of the system.

A number of measurements on a variety of materials have been made which show that the results with the glass electrode are fully as satisfactory as those obtained with the quinhydrone and hydrogen electrodes on the same materials.

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# A NOMOGRAM FOR USE IN CONNECTION WITH GUTZEIT ARSENIC DETERMINATIONS ON APPLES

W. J. YOUTEN

## INTRODUCTION

Two steps are usually involved in any quantitative observation. First, due care must be exercised in the selection of a sample that fairly represents the population under scrutiny, and second, adequate methods of evaluating the sample must be selected or devised. Three possibilities suggest themselves. The population may be so homogeneous or uniform that sampling difficulties are negligible. In other words, successive samples may be so nearly identical that the accuracy of the information gleaned may be dependent largely, if not practically entirely, on the precision of the method of examination of the sample. Or the reverse may be true. Here the system may be so variable that the selection of representative samples constitutes the major problem. Between these extremes is a middle ground where the investigator finds it necessary to consider both sample variation and the precision of the measuring procedure. The determination of the percentage of silver in coinage would probably fall in the first mentioned class. At least the question of sample selection would give less concern than the method of analysis adopted. The evaluation of the number of ounces of silver per ton of ore in a mining property illustrates the second group. The classification is not hard and fast, for, on the one hand, a new method of analysis may be developed which brings out differences between samples hitherto apparently identical, and on the other, increasing the sample size may reduce the variation among the samples to a point where the analytical errors are significant.

Suppose a number of observations have been made on a particular system, each observation for the moment being considered as one measurement on one sample. The mean and the probable error of a single observation are readily computed. If the system is homogeneous, that is of the first class mentioned, the computed probable error is simply the error of analysis. To return to the analysis of silver coinage, suppose one hundred observations have been made on United States quarters and another hundred on English shillings. Although the silver content of the coins of the two countries differs, the probable error found will agree closely for the two currencies provided the same method of analysis has been chosen. Consider now one hundred samples of ore taken from a mining property. If each sample is divided in two parts and a gravimetric method used on one set and a volumetric method on the other, then the mean and the *P.E.* of a single observation may be arrived at for each set. The two estimates of



the probable error will be in good agreement and approximate the probable error of a single sample. It is more common to find cases in which the probable error of a single observation must be ascribed to both the method of analysis and the technique of sampling. It is apparent that an understanding of the relative importance of the contributions of these two factors to the probable error of the recorded observations for any particular system is essential in order that the investigator may efficiently apportion his efforts between sampling and analytical methods.

It is the object of this paper to consider some of the previously published data obtained with the Gutzeit method on the arsenic content of apples as an illustration of the general case. On that account the statistical steps are given in greater detail than would be required to arrive at the conclusions for this case alone. The results are embodied in a nomogram which permits rapid estimation of the precision of a given procedure of sampling and analysis. The nomogram may be easily adapted to other situations.

#### THE $P. E.$ OF AN OBSERVATION AS A FUNCTION OF THE ERROR OF SAMPLING AND THE ERROR OF ESTIMATION

An observation was defined above as resulting from a single measurement on a single sample. If a number of observations  $O_1, O_2, O_3, \dots, O_n$  are available, then the mean,  $O_m$ , and the probable error of a single observation,  $(P.E.)_o$ , are given by the following formulas:

$$O_m = \frac{\sum O}{n} \quad (1)$$

$$(P.E.)_o = 0.6745 \sqrt{\frac{\sum d^2}{n-1}} \quad (2)$$

where  $\sum O$  symbolizes the sum of all the observations,  $n$  the number of observations, and  $d$  the difference between each observation and  $O_m$ .

The probable error of a single analysis,  $(P.E.)_a$ , may be arrived at by performing a number of analyses on a single uniform sample.

The probable error of a single sample,  $(P.E.)_s$ , is not usually susceptible to direct determination inasmuch as it would require an analytical method not subject to error. It is usually arrived at by computation although, in cases where the analytical error is small compared with sample variation, it may be taken as equal to  $(P.E.)_o$ .

Shewhart (8) in an article entitled "Correction of Data for Errors of Measurement" gives a quantitative relationship between these three probable errors.

$$(P.E.)_o^2 = (P.E.)_a^2 + (P.E.)_s^2. \quad (3)$$

The relationship assumes that the distribution of the samples approximates the normal error distribution.

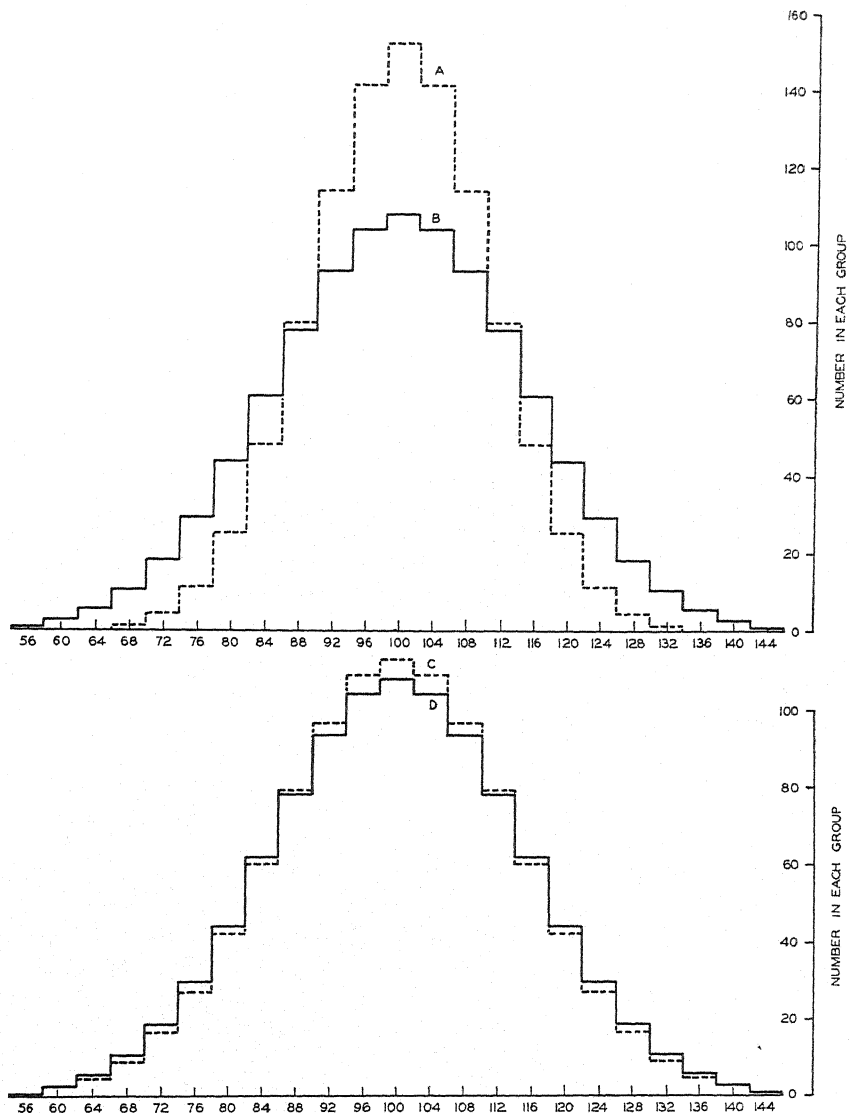


FIGURE 1. Histogram A shows the ideal distribution of 1000 samples where  $(P.E.)_s$  is 7.1 per cent. The samples are collected in groups, the group interval being 4 per cent of the mean. Histogram B shows the distribution of the observations made on the above samples where the  $(P.E.)_a$  is also 7.1 per cent of the mean. Note that the error of analysis has a marked influence on the observed distribution. Histograms C and D are similar to A and B. Histogram C is the expected distribution of 1000 samples where  $(P.E.)_s$  is 9.5 per cent. Histogram D is the distribution of the observations if the  $(P.E.)_a$  is 3 per cent. In this case the error of the analysis has little effect on the dispersion of the observations.

The character of the function may be ascertained by considering the ideal histograms shown in Figure 1. Each of the four histograms represents the distribution of 1000 observations, samples, or analyses. The class interval is 4 per cent of the mean and 100 is taken as the mean. The midvalue of each class interval is marked on the base line. The number of individuals in each class may be read from the scale at the right. Histogram A shows the distribution of 1000 analyses made on a uniform sample where the probable error of a single analysis is 7.1 per cent of the mean. The same histogram also indicates the distribution of 1000 samples where the probable error of a single sample is 7.1 per cent of the mean. If these samples are analyzed by a method whose probable error is 7.1 per cent, the final observations are distributed as in histogram B. The probable error of a single observation becomes 10 per cent of the mean. In this case both sample variation and analytical error contribute equally to the dispersion of the recorded observations.

Histogram C presents the expected sample variation for a group of 1000 samples with a probable error of a single sample of 9.5 per cent of the mean. Should these samples be examined by a method with a probable error of 3 per cent the resulting distribution of the observations is shown in histogram D. The probable error of a single observation is again 10 per cent of the mean. The predominating character of the sample variation is clearly evident and the spread of the observations but slightly greater than the actual sample distribution. Further refinement of the analytical procedure will contribute but little to the final precision. Should the situation be reversed and  $(P.E.)_a$  be 9.5 and  $(P.E.)_s$  be 3 per cent of the mean, then further care in sampling is practically wasted and attention must be directed to improving the mode of analysis or performing replicate determinations on each sample. It follows that a survey of this character should disclose, for example, whether, with current methods of selecting apple samples and the present Gutzeit method, it is desirable to improve the sampling or the analytical procedure.

Reference has been made to the fact that an estimate of  $(P.E.)_s$  is usually arrived at by computation. By rearranging Formula 3 in the form

$$(P.E.)_s^2 = (P.E.)_o^2 - (P.E.)_a^2 \quad (4)$$

it is evident that the  $(P.E.)_o$  and  $(P.E.)_a$  must first be obtained. Since the  $(P.E.)_a$  may be determined once for all for a particular analytical procedure it is a simple matter to estimate the character of any population from the recorded observations.

The formula is modified if duplicate or replicate analyses are conducted on each sample. Suppose that  $(P.E.)_a$  is known or determined from repeated analyses on the same sample. If duplicate analyses are conducted on each sample the probable error of the average of the two analyses be-

comes  $\frac{(P.E.)_a}{\sqrt{2}}$ . and the expression for the  $(P.E.)_o$  is written

$$(P.E.)_o^2 = 1/2(P.E.)_a^2 + (P.E.)_s^2.$$

In one of the papers consulted for data the author reported a number of observations each of which was a single analysis on a single sample. The same samples were also submitted to duplicate analyses. The probable error of a single observation was computed for each case. Let these be symbolized by  $(P.E.)_o$  and  $(\overline{P.E.})_o$ . We have the two equations

$$\begin{aligned}(P.E.)_o^2 &= (P.E.)_a^2 + (P.E.)_s^2 \\ (\overline{P.E.})_o^2 &= 1/2(P.E.)_a^2 + (P.E.)_s^2.\end{aligned}$$

Subtracting twice the second from the first there remains

$$(P.E.)_s^2 = 2(\overline{P.E.})_o^2 - (P.E.)_o^2 \quad (5)$$

and by direct subtraction

$$(P.E.)_a^2 = 2(P.E.)_o^2 - 2(\overline{P.E.})_o^2. \quad (6)$$

By this approach there is disclosed the nature of the population and of the analytical errors.

#### THE PROBABLE ERROR OF A SINGLE ANALYSIS

In 1929 Neller (6) published a study of the accuracy of the Gutzeit method and in 1930 (7) a paper on sampling. Both furnish data upon which an estimate of the probable error of a single analysis may be based. Barnes and Murray (2) have recently contributed an article on the accuracy of the analytical method.

Neller devotes especial attention to the probable error of the average of duplicate analyses on a single sample and usually computes it from the averages of duplicate analyses. In Table VI of his article (6) he gives his estimate of the  $P.E.$  of the mean of duplicate analyses as 6.6 per cent of the mean. This is based on 12 experiments averaging 10 determinations to the experiment. The  $P.E.$  of a single analysis is given in the same table as 10.0 per cent of the mean. If this figure is taken, the probable error of the average of duplicate analyses is immediately obtained by dividing by  $\sqrt{2}$ . The figure so obtained is 7.1 per cent. The arithmetical procedure used by Neller in arriving at the  $P.E.$  of the mean of duplicate analyses is incorrect. He suggests that from 10 replicates it is possible to form 45 different pairs and 45 means. From these 45 means an estimate of the probable error is made. This gives an erroneous result. Only five distinct pairs can be formed. An over-optimistic estimate of the probable error of a pair will inevitably

result through the seeming large increase of the number of pairs available. The present author was at some pains to resolve the tedious process of forming pairs, their means, and the final probable error of the means into a simple formula.

Suppose 10 observations have been made, then the *P.E.* of a single observation is given by the customary formula

$$0.6745 \sqrt{\frac{\sum d^2}{n-1}} \text{ where } n = 10$$

and the *P.E.* of the mean of a pair

$$\frac{1}{\sqrt{2}} 0.6745 \sqrt{\frac{\sum d^2}{n-1}}.$$

The use of the method of forming all possible pairs is equivalent to the use of the formula

$$\frac{1}{\sqrt{2}} 0.6745 \sqrt{\frac{\sum d^2}{n+1}}.$$

An inspection of the two formulas reveals that the process of forming all possible pairs is equivalent to replacing the factor  $(n-1)$  by  $(n+1)$  in the accepted formula. If  $n$  is large no great harm is done. Even if  $n$  is not less than 10 the *P.E.* is underestimated by 10 per cent and it is in just such cases where the labor of forming all conceivable pairs is not too onerous that the temptation to employ it has existed. This explains the fact that Neller, using 10 determinations to a set, computes the value 6.6 for the *P.E.* of the mean of a pair when his value of 10 for a single determination establishes the error of a pair as 7.1.

The paper by Barnes and Murray (2) is valuable from both chemical and statistical viewpoints. It very properly points out the part played by known standards used for comparison. If the analysis is compared with a standard which is based on 10 trials, the error will naturally be less than if the standard itself is the result of a single trial. Their figures show that the *P.E.* is reduced from 0.0039 to 0.0025 mg. It is also advanced that these errors are sensibly constant over the range from zero to 0.035 mg. For quantities at the upper end of their range the error of the analysis is from seven to ten per cent of the arsenic present. Through the use of properly proportioned aliquots this precision is attainable in practice and is in keeping with the other estimates.

#### THE PROBABLE ERROR OF SAMPLING

The examination of the literature for the purpose of disclosing the magnitude of sample variation is less satisfactory. Each fruit crop has its own history which determines the extent of sample variation. It will be instruc-

tive to compute the *P.E.* of a single sample in several instances and thus form an estimate of the limits within which the *P.E.* may reasonably be expected to fall.

A paper by Barnes (1) which gives the distribution of analyses on 299 individual unwashed apples affords a splendid opportunity to compute the *P.E.* of the arsenic content of a single apple. The mean arsenic content of one apple was found to be 0.031 grains/lb. with a *P.E.* of 0.0113. The *P.E.* of a single observation (one analysis on one apple) is therefore 36.5 per cent of the mean. Hence

$$(36.5)^2 = (P.E.)_s^2 + (10)^2.$$

Here the *P.E.* of a single Gutzeit analysis is taken as 10 per cent and

$$(P.E.)_s = 35.1 \text{ per cent.}$$

It is immediately seen that the distribution of the observations is practically that of the samples, the error of the analysis being relatively insignificant. Even if the analysis error were assumed to be as much as 15 per cent the sample error would be reduced but little, the figure being 33.3 per cent.

The diversity of the estimates in Table I was anticipated. The unwashed apples show the highest degree of variation. This seems to hold even though the arsenic content of the washed apples examined by Neller is not greatly different from that shown by the unwashed apples reported

TABLE I  
ESTIMATION OF VARIATION IN APPLE SAMPLES

Source	Washed or unwashed	No. apples in sample	No. of samples	Mean arsenic content grains/lb.	<i>P.E.</i> arsenic content single apple in % of mean
Barnes (1)	Unwashed	1	299	0.031	35
Hartzell and Wilcoxon (3)	"	1	24	0.0008	70
" " " (3)	"	1	23	0.0017	52
" " " (4)	"	4	12	0.0008	57
" " " (4)	"	4	8	0.0006	41
Heald, Neller, Overley, and Dana (5)	"	6	7	0.056	45
Neller (7)	Washed	6	10	0.019	13
"	"	6	10	0.017	0
"	"	6	10	0.019	22

by Barnes. Neller (7) in a paper just issued is inclined to the belief that the unwashed apples differ inappreciably from the washed as regards sampling error. This conclusion was based on Tables III and IV in his paper which were also used to illustrate that five samples offered no material advantage

over two. Neither contention stands examination. Neller used the scheme of taking the mean of five samples, *selecting* the two most divergent of the five, and comparing the mean of these two with the mean of the five. This artifice has no statistical value and only disguises the errors that are present. Contrary to the statement in the article the mean of two samples selected at *random* from the five will differ more, from the mean of the five, not less, than the mean of the two extreme samples.

A simple numerical illustration will point out the nature of the difficulty. Let 5, 6, 7, 8, 9 represent five analyses on washed apples and 3, 5, 7, 9, 11 the results of five analyses on unwashed (the average arsenic content is the same however). Notice that of the ten possible pairs that might be drawn in both cases there are two pairs in each case whose means agree exactly with the means of the groups, these are the extreme values and the pair bracketing the middle value; all the others differ. In addition, although the results on the unwashed apples show a much greater variation than the other set this difference in variation disappears when the mean of the extreme values in each set is compared with the mean of their group. Naturally in the case of actual data we do not have perfect symmetry with just five values, but the theory of errors does postulate such a symmetry and it is safe to predict that if much larger groups than five were used it would be quite impossible to distinguish at all between populations of the most different degree of variability by any such examination of the data. The illusion of accuracy created led to the conclusion in the paper (7) just mentioned that the use of duplicate samples reduced the percentage error from the 15.52 per cent found with single samples to 2.58 per cent. As a matter of fact, the error of the mean of duplicates may be found directly from the error of one sample by dividing by  $\sqrt{2}$ . In this case the figure of 11.0 per cent is more than four times the error reported by Neller.

The foregoing discussion shows that it is not possible to set a definite figure for sample variation. Suppose, for illustration, that it is assumed that the probable error of the arsenic content of a single apple is 40 per cent of the mean. A sample consisting of an aggregate of six apples would have a  $(P.E.)_0$  of  $40/\sqrt{6}$  or 16.3 per cent. If the error of a single analysis is 10 per cent, the  $(P.E.)_0$  is then

$$(P.E.)_0 = \sqrt{(10)^2 + (16.3)^2} = 19.2 \text{ per cent.}$$

If duplicate analyses are run on the sample

$$(P.E.)_0 = \sqrt{\left(\frac{10}{\sqrt{2}}\right)^2 + (16.3)^2} = 17.8 \text{ per cent.}$$

The gain is little more than one per cent. If the two analyses are performed on duplicate samples the probable error of the mean of the two observa-

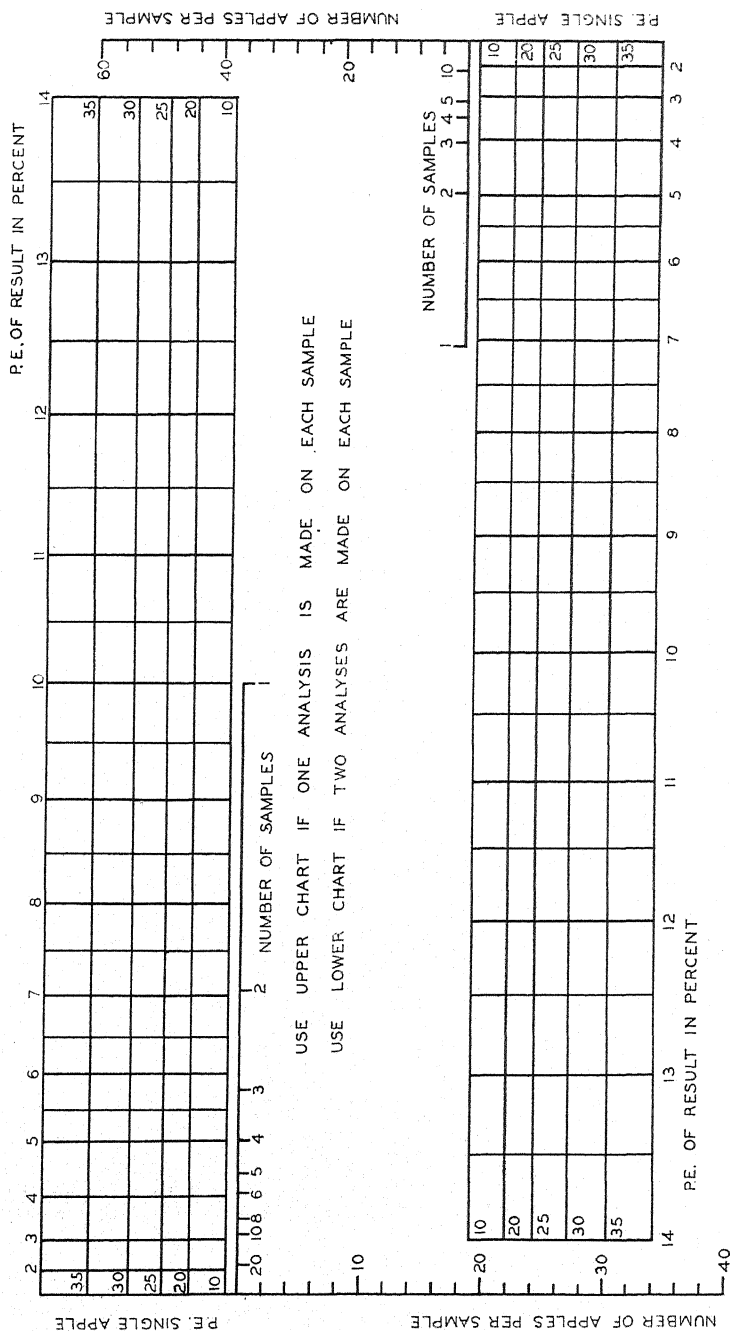


FIGURE 2. A nomogram showing the relation between  $(P.E.)_n$ ,  $(P.E.)_n$ , and  $(P.E.)_n$ . To use the above nomogram connect a point on the axis giving number of apples per sample with a point on the axis showing number of samples taken and extend the line determined by these points. At the intersections of this line with the lines marked 10, 20, 25, 30, and 35 (these lines represent the probable error of the arsenic content of a single apple in per cent of the mean) the corresponding probable error of the result in per cent may be read directly. The chart assumes that the error of a single Gutzeit analysis is 10 per cent.



tions is  $19.2 \div \sqrt{2}$  or 13.6 per cent. A much greater gain in accuracy results from two analyses on two samples. If the two samples are combined into one sample of 12 apples and one analysis made, the (*P.E.*)<sub>0</sub> is 15.3 per cent. This is a greater improvement over 19.2 than running duplicates on a six apple sample. The conclusion is drawn that with populations of the assumed degree of variability the employment of duplicate analyses on small samples is wasteful; rather attention should be paid to increasing the number of apples in the sample.

The uncertainty in the character of the apple population as compared with the analytical error led the author to prepare a nomogram (Fig. 2) from which may be read directly the probable error of the final observation for populations of a wide range of variability. The upper chart was prepared on the basis of one analysis per sample and assumes a probable error of a single analysis of 10 per cent of the mean. By laying a straight edge along the points representing the number of apples per sample and the number of samples taken, the *P.E.* of the result in per cent may be read for samples ranging from 10 to 40 per cent in probable error of the arsenic present. Thus, two samples of six apples may give a result with a probable error varying from 13.5 to 7.7 per cent as the probable error of the arsenic content of a single apple varies from 40 to 10 per cent. Also for any desired final error and assumed population various combinations of number of samples and apples per sample may be chosen. The lower chart differs only in that the error of analysis is taken as 7.1 per cent which is equivalent to taking the mean of duplicate analyses for every sample. The difficulty in selecting a safe value for the probable error of the arsenic content of a single apple cannot be minimized. Many factors contribute to the variation of the apple population. Among these are the ratio of surface to weight, waxiness, nature and frequency of application of the spray mixture, and the extent of the rainfall. The chart should be useful, however, in graphically depicting the changing importance of the analytical error and the variability of the sample for a wide range of conditions.

#### SUMMARY

An attempt has been made to clarify the factors involved in analyzing apples for their arsenic content. It was found that evidence had been previously presented which furnished a reliable estimate of the analytical errors. Sampling errors appear to vary greatly and almost certainly exceed the errors of analysis.

Certain statistical errors which marred earlier work have been discussed. These errors were stressed since they unfortunately tended to disguise the sampling error.

A nomogram has been prepared which serves as a convenient device for estimating the precision of the procedure adopted.

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## HERPETOMONAS BANCROFTI N. SP. FROM THE LATEX OF A FICUS IN QUEENSLAND

FRANCIS O. HOLMES

In 1927 Bancroft (1) reported the discovery of flagellates in the latex of four species of plants in Queensland, Australia. These plants were *Sarcostemma australe*, *Hoya australis*, and *Secamone elliptica* of the family Asclepiadaceae, and *Ficus scabra*, a wild fig of the family Moraceae. Intensive studies have been made of flagellates found in the latex of plants of the Euphorbiaceae and of the Asclepiadaceae, but flagellates from plants of the Moraceae have been mentioned only by Franchini (4, 5) who reported flagellates in *Ficus carica* and *F. parietalis* in France, by Fantham (2, 3) who found flagellates in *Ficus edulis* in South Africa, and by Bancroft (1) who found flagellates in *Ficus scabra* in Queensland as indicated above.

Bancroft kindly sent to the writer a great number of slides of infected and uninfected latex from the trees of *Ficus scabra*<sup>1,2</sup> among which he had found the infected individuals. Examination of the preparations of latex after staining showed that this Queensland flagellate could be distinguished at a glance from all other known herpetomonad flagellates by the presence of individuals in chains, such as are shown in the figures of this paper. These chains, consisting of 3 to 30 individuals, occurred in every preparation containing the flagellate.

The purpose of this paper is to describe the *Ficus scabra* flagellate and to record the available evidence bearing upon the manner of formation of the characteristic chains of cells.

Flagellate-infected specimens of *Ficus scabra* appeared to be rare, for, although Bancroft repeatedly sent fixed smears of latex from a number of individual plants, all the preparations which showed flagellates were from the latex of three trees growing near Eidsvold, Queensland. In these trees the flagellates were localized, being numerous in latex from some parts, and

<sup>1</sup> According to F. Manson Bailey, Comprehensive Catalogue of Queensland Plants, Brisbane, 1913, both *Ficus scabra* G. Forst. and *F. hispida* Linn. f. have been found in Queensland. In Index Kewensis *F. scabra* Jacq. is given as a synonym of *F. hispida* Linn. f. Presumably Bancroft's material was from *F. scabra* G. Forst.

<sup>2</sup> A letter kindly sent by C. T. White, Government Botanist at the Botanic Museum and Herbarium, Botanic Gardens, Brisbane, Queensland, received as this paper went to press, stated that he would have little hesitation in referring to *Ficus opposita* Miq. the Queensland Herbarium specimens, collected by Bancroft and determined by the late F. M. Bailey as *F. scabra* Forst. According to Bancroft's notes the tree is a small one common on the Burnett River, the largest tree about 20 feet high with a stem diameter of about nine inches and of very spreading habit. The leaves are mostly opposite and very scabrous-pubescent above.

absent from latex drawn from other points nearby. This localization naturally resulted in the absence of flagellates from some preparations. Unfortunately all latex preparations made directly from the trees proved upon examination in this country to have been from uninfected branches; only smears made at the laboratory in Queensland showed the organisms when examined here. These laboratory preparations were made from cut branches which were found by preliminary examination to contain the organisms. Others may discover this species in plants of the genus *Ficus* near their laboratories, and may be able to observe whether chains of flagellates are present when the latex is drawn from previously unwounded plants.

The flagellate in the latex of *Ficus scabra* was examined after the smears containing it were stained with Wright's stain. It proved to be of typical herpetomonad morphology, except for the presence of chains of individuals. The body was flat and ribbon-like, frequently twisted even in short cells in the manner which has been found characteristic of many latex flagellates. The nucleus was about one and one-half microns in length and a micron in width; its anterior end was 2.4 microns from the anterior end of the body, on the average. The parabasal body, spherical or nearly spherical in shape, was located near the middle of the space between the anterior end of the nucleus and the anterior end of the cell; in 122 observed cells variations from this position were equally common toward anterior and toward posterior ends.

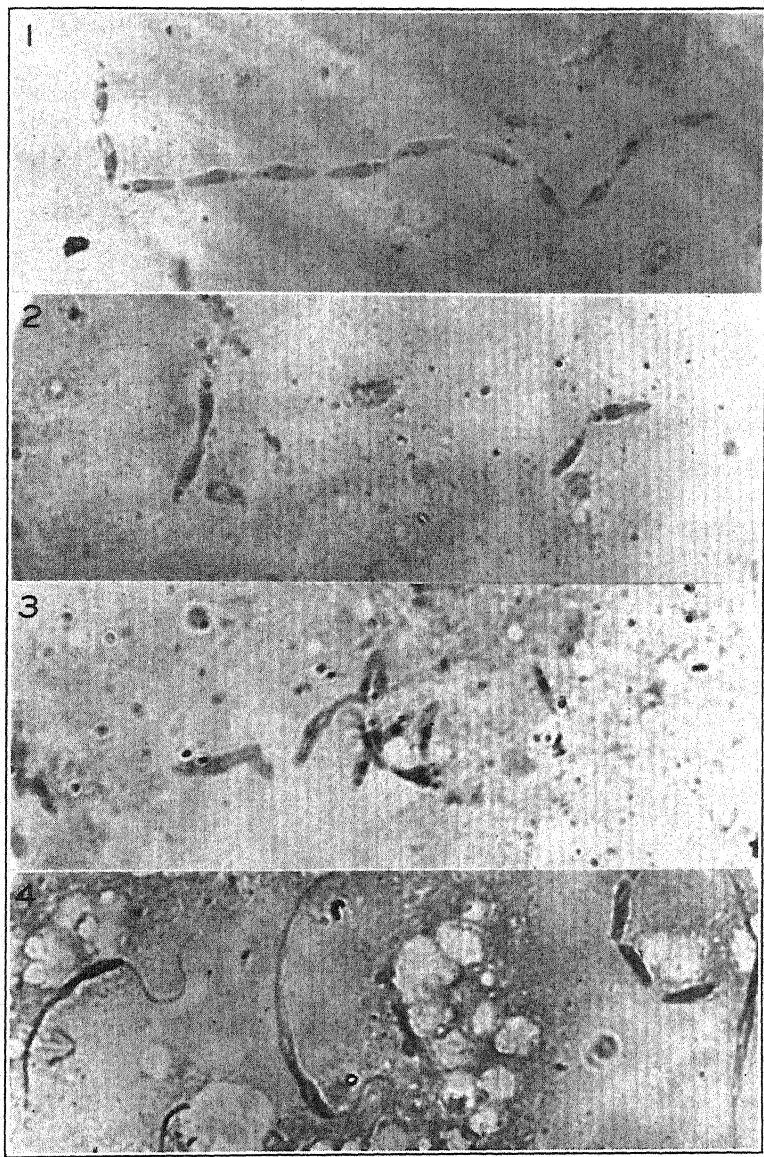
The lengths of the individuals in the various groupings are shown in Table I. The individuals in chains showed little variation and were com-

TABLE I

NUMBER OF INDIVIDUALS OF *FICUS SCABRA* FLAGELLATE OF EACH OBSERVED LENGTH

	Length of individual in microns																			
	5	6	7	8	9	10	11	12	13	14	15	16	18	19	20	21	22	23	24	
Isolated individuals	4	48	73	33	12	8	8	6	11	8	10	8	6	1	0	3	4	2	1	
Individuals in pairs		36	39	28	5	5	1			1	1				2					
In chains of 3		11	17	7	2	2														
In chains of 4		18	16	4	2															
In chains of 5		4	6																	
In chains of 6		3	1	2																
In chains of 7	2	7	5																	
In chains of 8			12	4																
In chains of 11				5	5	1														
In rosettes of 4		4	4	4																
Totals	6	131	173	87	26	16	9	6	11	9	11	8	6	1	2	3	4	2	1	

Total number of individuals observed: 512. Average length: 8.3 microns.  
Most frequent length: 7 microns.



FIGURES 1 to 4. *Herpetomonas bancrofti* n. sp. in latex of *Ficus scabra*. Magnification 1240 diameters. 1. Chain of 12 cells showing regular alternation of cell ends. 2. Two pairs of cells, one with posterior and one with anterior cell ends free. 3. Simultaneous division of three cells in a chain. 4. Long cells with flagella, and short cells in chain without flagella.

paratively short. The extreme range of lengths of individuals in all the chains recorded in the table was 5 to 10 microns. In contrast to this the range of lengths for isolated cells in the same table was 5 to 24 microns. Dividing pairs consisted mostly of short cells, but some pairs of relatively long cells, 11 to 20 microns in length, were observed.

The longer isolated cells showed flagella about eight-tenths as long as the body length, on the average. The longer paired cells showed flagella of nearly equal length on both daughter cells. Short cells, whether isolated, in pairs, or in chains, appeared to lack flagella. The flagella of long cells and the absence of flagella from short cells in chains are illustrated in Figure 4.

Resting cells were characterized by what appeared to be furrowing of the body, which looked thinner along its longitudinal center line than on its edges. The furrowing was especially conspicuous because the nuclei often appeared furrowed also. Examples of the effect of furrowing on nuclei are shown in Figure 5 in the 6th, 7th, 18th, 21st, 26th, and 27th cells of a long chain, counting the cells from the end near the top of the page.

Another characteristic of the *Ficus scabra* flagellate was the unusually common occurrence of pairs of cells. The frequency of appearance of these pairs may be connected in some way with the ability of the species to form chains of individuals. The numbers of aggregates of different sizes in a series of 512 specimens, constituting about one hundred flagellates from each of five latex preparations, were recorded. The totals were: 246 isolated

TABLE II

NUMBERS OF ISOLATED AND OF AGGREGATED INDIVIDUALS AMONG APPROXIMATELY 500 SPECIMENS OF EACH OF SIX LATEX FLAGELLATE SPECIES

Species	Isolated	In pairs	In chains of 3 or more	In rosettes
Flagellate from <i>Ficus scabra</i> <sup>1</sup>	246 or 48.0%	118 or 23.1%	136 or 26.6%	12 or 2.3%
<i>Herpetomonas elmassiani</i> (Migone) in latex of <i>Asclepias nivea</i> L.	474 or 94.8%	26 or 5.2%	0	0
<i>Herpetomonas ficuum</i> Fantham in latex of <i>Ficus edulis</i> <sup>2</sup>	498 or 99.6%	2 or 0.4%	0	0
<i>Herpetomonas davidi</i> (Lafont) in latex of <i>Euphorbia braziliensis</i> <sup>3</sup>	484 or 96.8%	16 or 3.2%	0	0
Flagellate from <i>Sarcostemma australe</i> <sup>4</sup>	498 or 99.6%	2 or 0.4%	0	0
Flagellate from <i>Hoya australis</i> <sup>5</sup>	486 or 97.2%	14 or 2.8%	0	0

Preparations from which these counts were made were kindly furnished by the following: <sup>1,4,5</sup>T. L. Bancroft of Eidsvold, Queensland; <sup>2</sup> Dr. H. B. Fantham, University of the Witwatersrand, Johannesburg, Transvaal, Union of South Africa; <sup>3</sup> the late Dr. Hideyo Noguchi, then of Rockefeller Institute for Medical Research, New York, N. Y. Names of plant hosts are given in the table in the form used by the donors.



FIGURE 5. Longest observed chain of cells of *Herpetomonas bancrofti* n. sp., consisting of 30 individuals. Photographed in two parts, with a few cells at middle of chain shown in both parts. Magnification 875 diameters.



individuals, 118 individuals in pairs (59 pairs), 39 individuals in chains of 3 (13 chains), 40 in chains of 4 (10 chains), 10 in chains of 5 (2 chains), 6 in one chain of 6, 14 in chains of 7 (2 chains), 16 in chains of 8 (2 chains), 11 in one chain of 11, 12 in rosettes of 4 (3 rosettes). From the totals it may be seen that a little more than half of the cells were arranged in some sort of aggregate, 266 cells being involved in aggregates as opposed to 246 appearing as isolated individuals. More than one-fifth of all the individuals observed were in pairs. The numbers of isolated individuals, pairs, and other aggregates were counted in the same way in preparations of several species of latex flagellate. The results are shown in Table II in comparison with the totals given above. The *Ficus scabra* flagellate showed a far higher proportion of pairs than any one of the other species.

Not only were there many pairs among the *Ficus scabra* flagellates, but the pairs differed in appearance in one respect from those of other species; they were frequently observed lying in straight lines, as in Figure 2, whereas pairs in other species almost always lie in such positions as to form acute angles between the individuals.

The most remarkable characteristic of the *Ficus scabra* flagellate was its ability to form chains of cells. These chains were often long, and were much more conspicuous under low powers of the microscope than were the isolated individuals. The cells in chains were slightly separated from adjacent cells, apparently because of moderate shrinkage during fixation. The separation is shown well in Figure 1.

In order to obtain large numbers of records of the occurrence of the cells in chains of different lengths, the observations on aggregates recorded above were supplemented by additional counts in which the occurrence of isolated individuals was disregarded. There were found altogether in the extended series of observations 1088 individuals arranged in the following aggregates: 201 pairs, 62 chains of three, 58 chains of four, 12 chains of five, 14 chains of six, 7 chains of seven, 8 chains of eight, and 1 chain of eleven. Examination of this series shows that although there were in general fewer instances of long chains than of short ones, there were more aggregates of even numbers of cells than would be expected. There were nearly as many of four as of three, more of six than of five, and more of eight than of seven.

Although no preparation of the flagellates failed to show chains of cells, the numbers of chains bore various relations to the numbers of isolated individuals. Occasional preparations contained many isolated individuals and few chains. One preparation containing few isolated individuals and many chains was studied. Perhaps the disturbance of the latex in making the preparations may have broken down the larger groups more in some cases than in others. Among 2906 individuals counted in the preparation showing many chains there were 183 isolated individuals; 490 individuals in pairs; 315 in chains of 3; 368 in chains of 4; 245 in chains of 5; 324 in

chains of 6; 154 in chains of 7; 216 in chains of 8; 126 in chains of 9; 130 in chains of 10; 99 in chains of 11; 48 in chains of 12; 39 in chains of 13; 14 in a chain of 14; 15 in a chain of 15; 32 in two chains of 16; 34 in two chains of 17; 18 in a chain of 18; 26 in a chain of 26; and 30 in a chain of 30. It will be noted that every length of chain from 3 to 18 was observed at least once in this preparation; that the longer chains were in general less common than the shorter; and that even-numbered chains were much in evidence. The most remarkable feature of this preparation was the contrast between the relatively small number of isolated individuals (compared with the numbers recorded in Table II) and the relatively large number of chains, particularly of long chains. Among the flagellates counted in this preparation isolated individuals constituted but 6.3 per cent; individuals in pairs 16.9 per cent; and individuals in chains of 3 to 30 cells 76.8 per cent of the total.

The latex preparations differed in concentration of cells from those in which the flagellates were so crowded as to touch each other to those which contained less than one cell or aggregate in each field under an oil-immersion lens. If chains were formed by chance aggregation of previously separated cells it might be expected that they would be rare in preparations in which the flagellates were widely separated. This was not found to be the case. The longest chain in the series just mentioned was so far from isolated cells, pairs, or other chains, that it was photographed in two sections and reproduced at a magnification of 875 diameters in Figure 5, because its length gave evidence to support the hypothesis that chains are not formed by chance contact of crowded cells, but by some biological process.

The alternation of cell ends in the chains was very regular, as may be seen in the examples shown in Figures 1 and 5, the posterior end of each individual touching the posterior end of its neighbor, and the anterior end of each flagellate touching the anterior end of a neighboring cell, except at the ends of the chain. Thus it appeared that whatever process produced the chain occurred with great regularity.

Although the alternation within the chain was always regular, the end cells were not always the ones expected from the fact that the usual method of division leaves anterior ends free. The vast majority of cells in pairs were found attached to each other at their posterior ends, as is commonly the case in other species of herpetomonads immediately after division, but some cells were joined at their anterior ends, with posterior ends free. These may have been dislodged from long chains by accidental breaks between posterior ends instead of between anterior ends. Among 446 pairs counted, the individuals of 86 pairs were joined in this unusual way. One pair of this type is shown in Figure 2, with a normal pair. The same position of terminal cells was found in 32 of 150 observed chains of 4; in 19 of 68 chains of 6; in 8 of 35 chains of 8; in 6 of 13 chains of 10; in the only observed chain of 18; and in the only observed chain of 30.

## DISCUSSION

The process of chain formation in the *Ficus scabra* flagellate is puzzling, for no other latex flagellate species shows such chains. The only aggregates in other species are pairs, rosettes produced by rapid and incomplete division, and false rosettes produced by sticking together of flagellum-ends. If dividing pairs had a tendency to stick together by their flagellum-ends, some aggregates in the form of chains might be formed, and in them alternation of ends of cells would be regular, but double false rosettes probably would be formed also. No double false rosettes have been observed among the *Ficus scabra* flagellates. Aggregation of dividing cells does not, therefore, seem to aid in explaining the formation of chains of cells in this flagellate.

The present study has not furnished adequate evidence to confirm any theory as to the manner of formation of chains of cells. Two hypotheses which have occurred to the writer may be of interest, however.

One hypothesis to explain the observed chain formation is suggested by the observation that *Herpetomonas elmassiani* (Migone) in the latex of *Asclepias nivea* L. has sometimes shown a tendency to start the division of the body at the posterior end of the cell instead of at the anterior end. This tendency results in partial splitting of the cell with the formation of a double posterior end before nuclear division. It might be supposed that a chain of four could be formed after the normal formation of pairs, if both cells of a pair should divide with their posterior ends splitting before their anterior ends. Such successive divisions beginning alternately at anterior and posterior extremities of terminal cells would account for chain formation, but in each chain of four the free ends of the terminal cells would both be posterior ends. Only 32 of 150 observed chains of four among the *Ficus scabra* flagellates were found to have this arrangement of cells, the remainder having anterior cell ends free. This hypothesis seems inadequate, therefore, to explain the formation of chains.

A second hypothesis involves the possibility that pairs or chains might divide further as units. If both individuals of a pair should become mature nearly simultaneously and should be about to divide again, the first cytoplasmic break might be expected to begin as usual at the flagellum-end of one cell of the pair. When new cells had been formed in this way, the second dividing cell would be subjected to two forces tending to cause it to divide. The first would be the normal internal tension causing the cell to tend to split at both ends. The second would be the pull at the posterior end due to movements of the newly formed cells derived from the division of the first cell of the original pair. The resultant of the two forces might cause a split to occur at the posterior end of the second dividing cell of the original pair, although either of the two forces might be unable to accomplish this result without the simultaneous action of the other. Once the second individual

of the pair became nearly split apart, the internal tension in this cell might be relieved and the movements of the divided individuals might then be unable to pull the cells further apart. The two new pairs of cells might thus remain attached in a chain of four. A subsequent similar division affecting all four individuals in this chain might account for the splitting of the chain of four to form a chain of eight. Accidental breaks in such long chains would supply the shorter groups of three, five, six, and seven. Accidental breaks would also account for occasional pairs, chains of four, and chains of six with posterior ends of terminal cells free. This hypothesis would require, and hence would account for, the more frequent occurrence of chains with anterior ends of terminal cells free than of chains with posterior ends free.

The attempt to confirm the hypothesis by searching for forms in chains showing division stages was indecisive because of the extreme rarity of such cases. Occasional single cells at the ends of chains were seen in early division stages. Three chains of three were found with individuals dividing simultaneously; one such chain is shown in Figure 3, since it seems to offer some support for this hypothesis that division of chains as units may occur.

Whatever may be the mechanism by which chains are formed, this herpetomonad flagellate from the latex of *Ficus scabra* is set apart from all known species of the genus *Herpetomonas* Kent by the fact that in latex smears it shows chains of cells in which the individuals regularly alternate in position. It is therefore suggested that the organism be known as *Herpetomonas bancrofti* n. sp. in honor of its discoverer, T. L. Bancroft, of Eidsvold, Queensland, who so kindly provided material for the present study.

Description of *Herpetomonas bancrofti* n. sp.: Herpetomonad flagellate found in latex of *Ficus scabra*<sup>3</sup> in Queensland, Australia, characterized by furrowed body, frequent occurrence in pairs, and unique arrangement in chains of 3 to more than 20 individuals. Individuals in chains vary from 5 to 10 microns in length; isolated individuals and those in pairs vary from 5 to 24 microns in length. Average length of flagellum about eight-tenths of body length; flagella of daughter cells in pairs nearly equal in length; individuals in chains and most other short individuals without flagella. Nucleus one and a half microns in length by one micron in width; its anterior edge 2.4 microns from anterior end of body on average. Parabasal body nearly spherical, near middle of space between anterior end of nucleus and anterior end of cell.

#### SUMMARY

Microscopic preparations of flagellates in the latex of *Ficus scabra*<sup>3</sup> were furnished to the writer by T. L. Bancroft of Eidsvold, Queensland, Aus-

<sup>3</sup> See footnotes on page 375.

tralia. The flagellates were found to differ from herpetomonad species previously described from latex plants mainly in the occurrence of pronounced longitudinal furrowing in cells before the division of the organelles, in very frequent occurrence of pairs of recently divided cells, and in the presence of chains of individuals. Chains of 3 to 30 individuals, with regular alternation of ends of the cells within each chain, were observed. Since chains of individuals have not been reported in any herpetomonad species, they are described in this instance in the hope that fig trees in other localities may yield similar flagellates in which the process of chain formation may be studied. The name *Herpetomonas bancrofti* n. sp. is suggested for the species.

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## AFTER-RIPENING, GERMINATION, AND STORAGE OF CERTAIN ROSACEOUS SEEDS

WILLIAM CROCKER AND LEILA V. BARTON

### INTRODUCTION

It has long been known that seeds of many rosaceous plants of the temperate zone require stratification at a low temperature, that is, a considerable period in a moist medium at a low temperature (14), to prepare them for germination. Davis and Rose (13) found for *Crataegus mollis* that the seeds after-ripen in the moist condition at about 5° C., that the embryos are dormant, and that the pericarps lengthen greatly the time necessary for after-ripening at the favorable temperature. In studies on the after-ripening and germination of seeds of several rosaceous plants, Crocker (8, 9, 10, 11, 12) found that both the time and the optimum temperature for after-ripening vary considerably with the different sorts. Most of his work will be discussed in connection with experiments on the various genera reported in this paper. He found (9) that Damson plum seeds after-ripen perfectly in stratification at 5° C. in 12 to 16 weeks, but poorly at 0° or 10° C. Removal of pericarps does not favor after-ripening or germination of Damson plum seed. The pericarps split most quickly at 15° C., although the seeds will not after-ripen at this temperature. Evidently then the splitting of the pericarp is not the essential part of after-ripening. These seeds do not germinate at the after-ripening temperatures but must be placed at a higher temperature for germination. He found (9) that French pear seeds, after being stored dry for five or six months, after-ripen, and germinate well at 0° or 5° C., and almost as well at 10° C.

The ability of rosaceous seeds to withstand dry storage is of great practical interest for in the case of species used for understock, such as *Prunus americana* Marsh, heavy crops sometimes appear only on alternate years which makes it desirable to carry over seeds for poor bearing years. Also, with rosaceous seeds that have a short after-ripening period, it is convenient to keep the seeds in dry storage until time for stratification in mid or late winter. In 1851, Fleischer (14) stated that rosaceous seeds retain their vitality in dry storage for considerable periods. Crocker (11) mentions the fact that seeds of *Rosa multiflora*, *Prunus americana*, and domestic apple withstand dry storage for months without marked fall in vitality and expresses the belief that this applies to most rosaceous forms.

The term stratification (11) refers to the old method of placing seeds and sand in successive layers and exposing the whole mass to low temperatures for the purpose of after-ripening the seeds in case of slow-germinating species, such as rosaceous seeds, or for holding seeds that will not withstand

drying (*Quercus*, *Castanea*, etc.) at a subminimal temperature and in a moist condition until the desired time for planting. At present it is customary to mix the seeds thoroughly with the moist medium and hold the whole mass at the desired temperature. It has been well established that the sole function of the stratification medium is to give the seeds the proper water and air supply and that with these maintained the determining factor for after-ripening is the temperature. This means that sand, peat, or soil, as long as they are free from injurious substances and give proper water and air supply, are equally good. Granulated peat is superior, in general, to other stratification media because it holds a large reserve of water and at the same time gives a good air supply. From this it is evident that stratification could be eliminated entirely and the seeds sowed at once in the seed bed if it remained at the required temperature for sufficient time to after-ripen the seeds.

The belief is common among practical growers and some scientists (9) still hold that freezing is necessary to give the proper after-ripening of dormant seeds that need stratification. All of the work cited above establishes beyond doubt that rosaceous seeds after-ripen best at temperatures a little above freezing and that freezing is unnecessary and is ineffective in promoting after-ripening.

#### METHODS

In stratifying seeds at low temperatures the following method has been used for experiments described in this paper. The seeds were thoroughly cleaned and mixed with granulated peat that had been thoroughly wetted and then squeezed between the hands until all possible water was removed. The mixture of peat and seeds was placed in large-mouthed bottles, covered with cheesecloth, and put into ovens which were kept in a cold room (about  $-15^{\circ}$  C.) and electrically heated and regulated at the desired temperatures. The peat containing the seeds was poured from the bottle periodically and examined for germinated or moldy seeds. The germinated seeds were removed and counted and the moldy ones thoroughly cleaned and returned. The stratification mixture was then watered and returned to bottles which were replaced in the proper constant temperature chamber. After various periods of stratification the desired number of seeds were removed for planting in flats in a greenhouse or in cold frames outside. In case the complete germination was to be followed at low temperatures, the germinating seeds in the mixture were counted periodically until germination ceased.

When plantings were made in cold frames, either in the fall or mid-winter, the seeds were planted in flats and the flats were placed in cold frames. One of the cold frames was left without any cover, another was provided with a tight board roof, and the third was provided with a mulch

of leaves three to six inches deep and covered with a tight board roof. The seeds in the open frame had a fluctuation in temperature corresponding to the outdoor temperature, the seeds in the board-covered frames experienced less fluctuation in temperature but froze and thawed as the temperature changed, while the seeds in the mulched and board-covered frame fluctuated little in temperature and were always held somewhat above the freezing temperature.

#### EXPERIMENTAL RESULTS

*Apple seeds.* In a sound Borsdorfer apple that had been cellar-stored for nine months, Ascherson (3) found a seedling 35 mm. long. He spoke of this as an unusual phenomenon. Arthur (2) reports the finding of two young seedlings of *Pyrus coronaria* under a stone of the foundation of a barn which was removed twenty years after it had been built. Twenty days elapsed between the removal of the barn and the foundation. He believed that the seeds had lain dormant for twenty years and had germinated when furnished the proper moisture and temperature conditions.

Harrington and Hite (15) found apple seeds incapable of germination without after-ripening. Even when the embryos were excised before after-ripening only a few showed growth and those did not form normal seedlings. After-ripening of seeds did not occur in dry storage but there was after-ripening and some germination in moist conditions in an ice box at 5° to 10° C. after three to four months. Apple seeds also after-ripened and a small per cent germinated in the apples stored at 0° C. for several months. The authors pointed out that oxygen which is necessary for after-ripening and germination was abundant in cold storage apples (16). After apple seeds had been in a germinator for six months at temperatures too high for after-ripening they would germinate promptly and vigorously if all coats were removed, but not otherwise. Seeds after-ripened at 5° to 10° C. germinated promptly with coats intact. After-ripened seeds germinated best between 10° and 20° C., but 25° C. was too high. Apple seeds that had been dormant in germinators above 10° C. for five months showed a four-fold increase in respiration intensity when the outer coats were removed. They germinated when the inner coats were removed. The authors concluded that the coats play an important rôle in after-ripening and germination of apple seeds.

Pammel and King (17) found that seeds of *Pyrus ioensis* (Wood) Bailey would germinate in a greenhouse after they had lain on the ground under the trees during the winter, also seeds of *Pyrus malus* var. Jonathan (18) germinated promptly in a greenhouse after they were stratified outside during the winter.

Bakke, Richey, and Reeves (4) agree with Harrington and Hite (15) that apple seeds are dormant and need after-ripening and that the dor-



mancy is located in the embryos. They stored fresh apple seeds in moist chambers and fresh apple seeds that were first dried in desiccators at the following temperatures: room temperature,  $5^{\circ}$  to  $10^{\circ}$  C.,  $1^{\circ}$  to  $3^{\circ}$  C.,  $0^{\circ}$  to  $2^{\circ}$  C., and variable temperatures  $-25^{\circ}$  to  $+10^{\circ}$  C. After storage for various periods under these conditions, the seeds were planted in flats in a greenhouse that ran at  $10^{\circ}$  to  $17^{\circ}$  C. All the seeds stored dry in these conditions gave little or no germination. Moist storage at  $1^{\circ}$  to  $3^{\circ}$  C. gave best germination, moist storage at  $5^{\circ}$  to  $10^{\circ}$  C. good germination, and moist storage at  $-2^{\circ}$  to  $0^{\circ}$  C. fair germination. The other moist storage conditions were unfavorable for germination. The authors also conclude from meager data that seeds in dry storage for one year lose entirely their power to germinate. The dry stored seeds were apparently not after-ripened by storage in a moist condition at low temperature previous to planting. The failure of these seeds to germinate may have been due to dormancy rather than to lack of vitality. This paper will furnish evidence for this interpretation. In a summary article based on the data reported in this paper, Crocker (10) states that apple seeds show little degeneration with two and one-half years of dry storage but that the seeds must be properly after-ripened by stratification at a low temperature ( $0^{\circ}$  to  $10^{\circ}$  C.) for two or two and one-half months to prepare them for germination.

The apple seeds used in Tables I and II were furnished by M. B. Davis of the Dominion Experimental Farms of Ottawa, Canada. The seeds consisted of separate collections of 1924, 1925, and 1926 of the varieties Wealthy and Patten Greening (*Pyrus malus* L.), and *Pyrus baccata* L. hybrid seeds. After gathering of the fruits the seeds were removed, cleaned, dried, and stored in paper packets in the laboratory at the Dominion Farm until they were shipped to this Institute late in December of 1926. The experiments were started on February 15, 1927. This means that the 1924 seeds were held in dry storage about two and a half years, the 1925 seeds about one and a half years, and the 1926 seeds about one-half year.

Table I shows the after-ripening and germination of the three varieties of apple seeds mentioned above, including the 1924, 1925, and 1926 crops. One hundred seeds of each variety and harvest were mixed with properly moistened granulated peat (pH about 4), granulated peat that had been thoroughly leached to bring it temporarily near the neutral point, and granulated peat limed sufficiently to bring it permanently near the neutral point. The behavior of the seeds in the three media was so similar that the three are reported together giving the average per cent germination for 300 seeds of each lot in each temperature. The mixture of seeds and granulated peat was placed in ovens at  $1^{\circ}$ ,  $5^{\circ}$ , and  $10^{\circ}$  C., and in an ice chest varying between  $5^{\circ}$  and  $10^{\circ}$  C. The seeds after-ripened and germinated at all these low temperatures. The temperatures  $1^{\circ}$ ,  $5^{\circ}$  C., and ice chest were about equally effective in all three varieties and ages of seeds, while  $10^{\circ}$  C.

TABLE I

EFFECT OF VARIOUS LOW TEMPERATURES UPON THE GERMINATION OF THREE VARIETIES OF APPLE SEEDS OF VARIOUS AGES. SEEDS CLEANED AND KEPT IN DRY STORAGE FROM DATE OF HARVEST UNTIL EXPERIMENT WAS STARTED IN FEBRUARY 1927

Temperature	Year of harvest	Percentage of germination in 3 1/2 months		
		Wealthy	Patten Greening	<i>Pyrus baccata</i> hybrid
1° C.	1924	63	34.6	68.6
	1925	77	34.0	64.0
	1926	82	55.0	96.6
5° C.	1924	31	47.3	80.0
	1925	56	47.3	60.6
	1926	73	44.0	87.6
Icebox	1924	69	46.0	63.5
	1925	51	75.6	54.6
	1926	77	86.0	59.3
10° C.	1924	8	22.6	11.0
	1925	11	32.0	17.0
	1926	14	46.0	21.0

was much less effective. The two and one-half year old seeds showed almost as good germination as the one and one-half year old seeds but considerably poorer germination than the one-half year old seeds.

The maximum germinations at any of the favorable temperatures for Wealthy seeds were for the 1924 crop—69 per cent; 1925 crop—77 per cent; and 1926 crop—82 per cent; for Patten Greening, 1924 crop—47.3 per cent; 1925 crop—75.6 per cent; and 1926 crop—86 per cent; and for *Pyrus baccata* hybrid, 1924 crop—80 per cent; 1925 crop—64 per cent; and 1926 crop—96.6 per cent. The average germination at the three favorable temperatures was for Wealthy seeds, 1924 crop—54 per cent; 1925 crop—61.3 per cent; and 1926 crop—77.3 per cent; for Patten Greening, 1924 crop—42.8 per cent; 1925 crop—52.3 per cent; and 1926 crop—61.6 per cent; and for *Pyrus baccata* hybrid, 1924 crop—70.6 per cent; 1925 crop—59.7 per cent; and 1926 crop—81.2 per cent. From this it is evident that the germination of the 1924 seeds is only slightly below that of the 1925 seeds, the grand average for all varieties and favorable temperatures being 56 and 58 per cent respectively. The 1926 crop showed considerably higher germination, giving a grand average of 73 per cent. How much of this difference in the germination of the three years' crop is due to the original vitality of the seeds and how much to degeneration in storage can not be determined, but at any rate apple seeds that have been in dry storage two and one-half years still retain a very considerable germinating capacity.

Seeds of Wealthy, Patten Greening, and *Pyrus baccata* hybrid of the three crops 1924, 1925, and 1926 were mixed with moist granulated peat

on February 15, 1927, and placed at four different temperatures, 1°, 5°, and 10° C., and in an ice box fluctuating between 5° and 10° C. The first lot of these seeds, 100 of each sort and treatment, was taken out of stratification on March 15, after one month of stratification, planted in flats, and placed in cold frames with board covers at first and later, when seedlings began to come up, with glass or lath covers as conditions demanded. The second lot of these seeds, 100 of each sort and treatment, was taken out of stratification on May 1, after two and one-half months of stratification, and planted in flats which were placed in cold frames protected with glass or lath covers.

The results of this experiment are reported in Table II. In most cases one month of stratification gave much poorer results than two and a half

TABLE II

EFFECT OF STRATIFYING APPLE SEEDS OF VARIOUS AGES FOR VARIOUS TIMES AT FOUR DIFFERENT LOW TEMPERATURES. STRATIFIED FEBRUARY 15, 1927; 100 SEEDS EACH PLANTED IN FLATS IN COLD FRAMES AFTER ONE MONTH'S STRATIFICATION (MARCH 15) AND AFTER TWO AND ONE-HALF MONTHS' STRATIFICATION (MAY 1); SEEDLINGS COUNTED JULY 1, 1927

Year of harvest	Stratification temperature °C.	Period of stratification in months	Per cent seedling production by July 1		
			Wealthy	Patten Greening	<i>Pyrus baccata</i> hybrid
1924	1	1	20	13	15
	1	2.5	50	29	33
	5	1	6	20	19
	5	2.5	56	59	41
	10	1	Lost	Lost	7
	10	2.5	12	28	1
	Icebox	1	12	9	21
	Icebox	2.5	56	31	21
1925	1	1	37	17	26
	1	2.5	35	45	56
	5	1	4	31	11
	5	2.5	65	55	62
	10	1	Lost	1	0
	10	2.5	14	42	6
	Icebox	1	12	9	4
	Icebox	2.5	39	49	65
1926	1	1	45	63	42
	1	2.5	68	78	69
	5	1	12	59	30
	5	2.5	75	76	47
	10	1	1	21	1
	10	2.5	Lost	13	11
	Icebox	1	7	23	30
	Icebox	2.5	65	64	63

months' stratification in spite of the fact that, in the main, a low though widely fluctuating temperature existed in the cold frame for a month or more after the seeds were planted. In this experiment, as in the previous

one, 1°, 5° C., and ice box were much better temperatures for after-ripening than 10° C. After stratification at 5° C. for two and one-half months, the 1924 crop gave the following seedling production: Wealthy 56 per cent, Patten Greening 59 per cent, and *Pyrus baccata* hybrid 41 per cent. The 1925 crop gave 65, 55, and 62 per cent seedling production respectively, and the 1926 crop 75, 76, and 47 per cent seedling production respectively. As in Table I, the 1926 seeds gave, on the average, the highest per cent seedling production, the 1925 seeds next, and the 1924 seeds the lowest, but even with the 1924 seeds the seedling production was very good. For the best conditions of stratification they gave what in practice would be considered a perfect stand. The good quality of the two and one-half year old seeds was further proved by the fact that on July 1 when the count was made, the seedlings in all three harvests were of about equal size and of good growth and vigor. Here again it is not possible to state to what degree the fall in seedling production with age is due to the original vitality, and to what degree to the age of the seeds, but apple seeds stored dry for two and one-half years are capable of producing a good stand of vigorous seedlings.

More than half of the apple seeds taken from pomace at the cider mill after they have gone through the cutters and press show visible injury and even the seeds that show no visible injury and appear plump and fresh give only moderate to poor germination. Table III, collection I, shows the course of germination of seeds of domestic apples taken from the pomace

TABLE III

GERMINATION AT 5° C. OF DOMESTIC APPLE SEED, COLLECTED FROM POMACE, FALL OF 1925; STORED DRY UNTIL FEBRUARY 1926 WHEN EXPERIMENT WAS STARTED

Collection	Medium	Treatment	Per cent germination after				
			11 wks.	13 wks.	15 wks.	17 wks.	19 wks.
I	Peat	Uspulun treated	6.0	14.0	24.0	34.0	38.0
	Sand	Uspulun treated	4.0	12.0	12.0	38.0	40.0
	Peat	Not treated	4.0	14.0	24.0	40.0	42.0
	Sand	Not treated	4.0	8.0	16.0	38.0	46.0
II	Sand	Uspulun treated and changed to peat after 9 wks.		5.5	15.5	22.5	27.0

where only apparently sound seeds were used. Even after 19 weeks at 5° C. the germination had reached 38, 40, 42, and 46 per cent in the various lots. After 15 weeks, approximately three and one-half months, the percentage germination was 24, 12, 24, and 16 per cent respectively in the various lots. This is far below the percentage germination shown by two and one-half year old apple seeds from Canada with three and one-half months' germination at 5° C., as reported in Table I.

Table III, collection II, shows the results obtained with another batch of apple seeds taken from pomace in the fall, stored dry, and put to germinate at 5° C. in February. The sterilized seeds in this case gave only 27 per cent germination after 19 weeks, while the seeds that were not sterilized rotted without any germination.

It is not known to what extent the apparently good seeds are injured by the cutting or pressing or to what extent the fermentation or lack of oxygen in the pomace reduces the vitality.

Apple seeds that are removed from the fruit by hand and cleaned generally give good germination. Table IV shows the percentage germination for seeds of *Pyrus malus* L. var. Baldwin taken from cold storage fruit on February 24. Lot 1 was put into moist peat at 5° C. immediately after re-

TABLE IV

EFFECT OF ONE MONTH'S DRY STORAGE ON BALDWIN APPLE SEEDS TAKEN FROM TABLE APPLES THAT HAD BEEN IN COLD STORAGE UNTIL FEBRUARY 24

Lot No.	Per cent germination after being in moist peat at 5° C.					
	2 wks.	3 wks.	4 wks.	5 wks.	7 wks.	10 wks.
1	9	57	—	78*		
2	—	—	9	67	80	83**

\* 22 per cent lost by decay.

\*\* 17 per cent lost by decay.

moval and cleaning. In two weeks they gave 9 per cent germination, 57 per cent after three weeks, and 78 per cent after five weeks. They had completed their germination before dry stored seeds would begin germination in the same condition. This confirms the findings of others (3, 15) that apple seeds after-ripen in the fruit in cold storage. It is not unusual to observe germinated seeds in apples that have been in cold storage for six or seven months. The cold storage furnishes the temperature both for after-ripening and for germination as is indicated by the data given in this paper. The long period required for after-ripening and germination of the seeds in the cold storage apples indicates that either the temperature or the water supply or both are not optimum. Lot 2 was allowed to dry for one month in the laboratory after the removal from the fruit and cleaning. When they were then placed in moist peat at 5° C., germination started after four weeks with 9 per cent germination. At five weeks it had reached 67 per cent, and after seven weeks 80 per cent. Apparently the month of dry storage slowed down the initiation of germination slightly but germination was completed in almost as short a period as with the seeds that were not dried, and in a very much shorter period than is the case with seeds taken out of the fruit at harvest time and stored dry. The after-ripened condition, in the main, is retained even during a period of dry storage.

The most practical way for breeders and nurserymen to produce apple seedlings is to remove the seeds from the fruits immediately after harvest, clean them thoroughly, and store dry until time for stratification, then stratify the seeds in moist granulated peat at the proper temperature for two to two and one-half months and sow in cold frames or beds outside. Stratification at harvest time will give full germination in mid-winter when it is impossible to plant outside. Fall sowing outside may lead to germination at warm periods in the winter followed by freezes that kill the seedlings. One case was called to our attention where the breeder, assuming that apple seeds were killed by drying, stratified his hybrid seeds immediately after harvest. They germinated in stratification in mid-winter and were lost due to lack of a greenhouse in which to grow the seedlings.

*Peach seeds.* Crocker (9) has shown that peach seeds after-ripen and germinate well in stratification at 5° C., that 10° C. is less favorable, and 15° and 20° C. constant, and weekly alternations between -10° C. and 10° C., give no after-ripening or germination. He has also shown that removal of pericarps greatly hastens the after-ripening and germination of peach seeds.

The peach seeds (*Prunus persica* (L.) Stokes. varieties Elberta and Lemon Free) used in the experiments reported in Table V were furnished by W. H. Upshall of the Horticultural Experiment Station, Vineland Station, Ontario, Canada, and were shipped in October 1930.

In Table V it is seen that the seeds of Elberta peach germinated in three and one-half months at 1°, 5°, and 10° C. In all cases the removal of

TABLE V  
GERMINATION OF PEACH SEEDS AFTER THREE AND ONE-HALF MONTHS AT CONSTANT TEMPERATURES

Temperature	Medium	Pericarp	Elberta		Lemon Free	
			No. of seeds	Per cent germination	No. of seeds	Per cent germination
1° C.	Peat	Intact	125	11.2	225	12.8
		Removed	200	62.0	365	18.9
5° C.	Peat	Intact	225	42.6	325	28.9
		Removed	200	80.5	425	87.0
10° C.	Peat	Intact	225	40.8	225	40.4
		Removed	200	87.5	425	40.9
Greenhouse 70° F.	Soil + sand + peat	Intact	100	0	100	8.0
		Removed	57	15.8	100	6.0

the pericarp gave a higher percentage germination, but even with the pericarp intact 42.6 per cent germinated at 5° C. against 80.5 per cent with pericarp removed and at 10° C. 40.8 per cent with pericarp intact against 87.5 per cent with pericarp removed, while at 1° C. only 11.2 per cent germinated with pericarp intact against 62 per cent with the pericarp removed. In the greenhouse at 70° F. none germinated in the same time with pericarps intact and only 15.8 per cent germinated with the pericarps removed. For the Elberta 5° and 10° C. seemed very favorable for germination, while 1° C. was much less favorable. Lemon Free seeds showed similar behavior at 1°, 5°, and 10° C., especially if one allowed for the marked tendency of these seeds to rot when removed from the pericarp. The Lemon Free showed some germination in the greenhouse even with the pericarp intact.

When peach embryos are entirely removed from the coats and placed on moist filter papers at room temperature, more than 50 per cent will produce both roots and epicotyls within two weeks, showing that the embryos are less dormant than is the case with many rosaceous seeds. There is an increase in rate of growth of peach embryos as well as a larger percentage germination, however, when the seeds have been after-ripened in a low temperature germinator.

*Pyrus seeds.* Adams (1), working with seeds of *Aronia melanocarpa* (Michx.) Britton, obtained 15 per cent germination after 621 days when 20 freshly harvested seeds were planted outside in September. Seeds which he planted in the greenhouse immediately after harvest or kept dry out-of-doors or in a heated room and planted the following spring gave no germination. These results proved the existence of a period of dormancy and pointed to the need of low temperature for after-ripening.

Seeds of *Pyrus arbutifolia* (L.) L.f. and *P. arbutifolia* var. *atropurpurea* (Britton) Robinson were collected in New Jersey on October 7, 1928. Some of them were cleaned and put to germinate at once. Samples of 100 seeds each were put on moist filter paper at constant temperatures of 15°, 20°, 25°, and 30° C., and at daily alternating temperatures of 10° to 30° C., and 20° to 30° C. At the same time a planting was made in soil in a greenhouse flat. These tests were continued for five months but no seedlings appeared.

In an effort to break the dormancy of the seeds of these two varieties of *Pyrus*, they were given low temperature stratification at 1°, 5°, and 10° C. for periods of two, three, or four months. At the end of each of these periods samples of 50 seeds each for *P. arbutifolia* and 25 seeds each for *P. arbutifolia* var. *atropurpurea* were removed from the peat and planted in flats in a mixture of soil, sand, and peat. The flats were then kept in a greenhouse with a temperature of 68° to 70° F. Here, seedlings began to appear after 8 to 20 days, and the seedling production was complete within 20 to 30 days.

It will be seen from Table VI and Figure 1 that either 1° or 5° C. is a suitable temperature for the after-ripening of seeds of *Pyrus arbutifolia*,

TABLE VI  
SEEDLINGS PRODUCTION FROM SEEDS OF PYRUS IN THE GREENHOUSE AFTER STRATIFICATION  
AT LOW TEMPERATURES FOR 2, 3, OR 4 MONTHS

Species	Stratification temperature ° C.	Peat				Neutral peat		
		Per cent germination after stratification for				Per cent germination after stratification for		
		Check	2 mos.	3 mos.	4 mos.	2 mos.	3 mos.	4 mos.
<i>P. arbutifolia</i>	1	0	70.0	100.0	84.0	64.0	88.0	78.0
	5	0	36.0	94.0	*	14.0	92.0	*
	10	0	0	0	4.0	0	0	4.0
<i>P. arbutifolia</i> var. <i>atropurpurea</i>	1	0	92.0	44.0	37.0	—	—	—
	5	0	12.0	64.0	36.0	—	—	—
	10	0	100.0	4.0	8.0	—	—	—

\* Not planted.

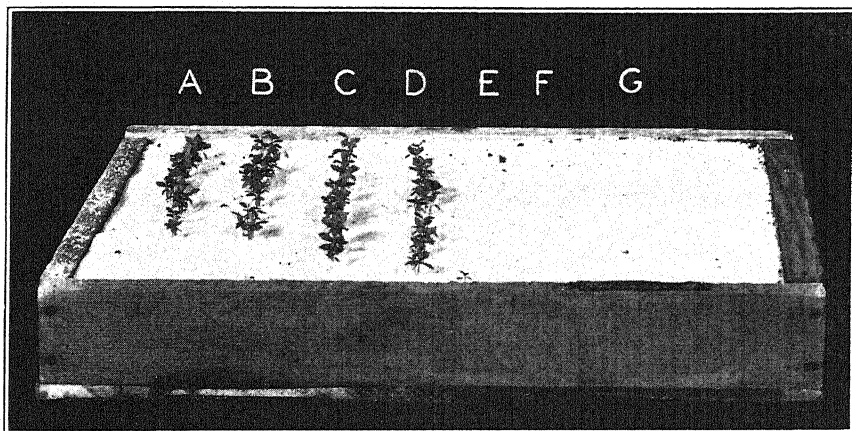


FIG. 1. *Pyrus arbutifolia* seeds stratified three months, then planted in a flat in a 70° F. greenhouse. Stratification condition: A, in peat at 1° C.; B, in neutral peat at 1° C.; C, in peat at 5° C.; D, in neutral peat at 5° C.; E, in peat at 10° C.; F, in neutral peat at 10° C.; G, planted without stratification. Picture taken 29 days after planting in the greenhouse.

while 10° C. is ineffective. A seedling production of 100 and 94 per cent was obtained from the greenhouse planting of seeds which had been exposed to temperatures of 1° and 5° C. respectively for three months prior to planting. The same period of stratification at 10° C. gave no seedlings. Dry seeds planted as controls produced no seedlings.



It will be noticed that three months at  $1^{\circ}$  C. gave better results than either two or four months at  $1^{\circ}$  C. Three months was also the optimum stratification time at  $5^{\circ}$  C. Two months gave less than half as many seedlings while at the four-month period so many seeds had germinated at the low temperatures that it was impractical to make greenhouse plantings. Hence it is seen that time as well as temperature is a very important factor in the after-ripening of dormant seeds.

*Pyrus arbutifolia* var. *atropurpurea*, on the other hand, responded well (92 and 100 per cent germination in the greenhouse) after having been in temperatures of  $1^{\circ}$  and  $10^{\circ}$  C. respectively for a period of two months. A temperature of  $5^{\circ}$  C., which would be expected to give better results than  $10^{\circ}$  C., and as good as  $1^{\circ}$  C., was not effective at this period (Table VI). After three months' stratification, however, seeds from  $5^{\circ}$  C. germinated to the extent of 64 per cent, while those from  $1^{\circ}$  and  $10^{\circ}$  C. showed marked decreases in seedling production at this period. Again, the stratification time proves to be an important factor. Material was not available to retest the effect of stratification at  $5^{\circ}$  C. so it is not known whether the poor results after two months' stratification were due to a mishap in the handling or to the low effectiveness of this temperature. It is likely the former.

Since the peat used had an acid reaction, it was thought that a different response might be obtained if the peat were neutralized with powdered calcium carbonate. Hence a series of tests was made with seeds of *Pyrus arbutifolia* in which neutral peat was used as the stratification medium. There were no marked differences in the results (Table VI and Fig. 1).

*Amelanchier* seeds. Seeds of *Amelanchier canadensis* (L.) Medic. were obtained from a seedsman in October, 1928. They were cleaned immediately and divided into two parts, one of which was left to dry for three months in the laboratory while the other was used for experiments which were started at once.

The seeds were mixed with moist granulated peat and put at low temperatures ( $1^{\circ}$ ,  $5^{\circ}$ , and  $10^{\circ}$  C.). Here they were allowed to remain for periods of three, four, and five months. At the end of each of these periods samples of 100 seeds each were removed from the peat and planted in the greenhouse in a mixture of peat, sand, and soil.

The same types of tests were made with the seeds which were allowed to dry for three months except that in this case sample plantings were made in the greenhouse after two and three months of low temperature treatment.

A reference to Table VII will show the results obtained. It will be seen that previous stratification at  $1^{\circ}$  or  $5^{\circ}$  C. for three or four months in the case of the fresh seeds (Fig. 2) or at the same temperatures for three months in the case of seeds which had been dried for three months gave the best seedling production in the greenhouse. After four months the fresh

TABLE VII

GERMINATION OF FRESH AND DRIED SEEDS OF *AMELANCHIER CANADENSIS* IN THE GREENHOUSE AFTER STRATIFICATION AT LOW TEMPERATURES FOR 2, 3, 4, OR 5 MONTHS

Stratification temperature °C.	Fresh seeds			Dried 3 months	
	Per cent germination after stratification for			Per cent germination after stratification for	
	3 mos.	4 mos.	5 mos.	2 mos.	3 mos.
1	14	21	3	5	38
5	19	26	6	1	28
10	0	0	0	0	0
Check	0	0	0	0	0

seeds started to germinate at the low temperature. This accounts for the poor germination after five months' stratification. The dried seeds began

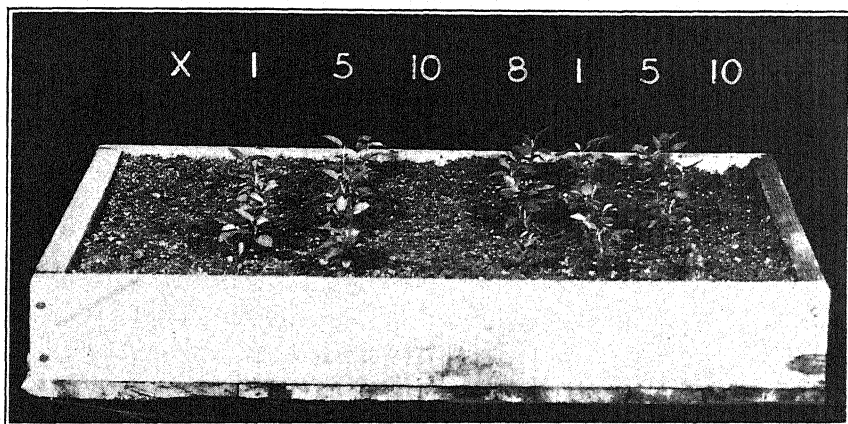


FIGURE 2. *Amelanchier canadensis* seeds stratified before planting in flat in greenhouse at 70° F. X, check of dry seeds. Picture 67 days after planting. Four at left: seeds stratified at 1°, 5°, 10°, and 8° C. three months before planting. Picture 67 days after planting. Three at right: seeds stratified at 1°, 5°, and 10° C. four months before planting. Picture 37 days after planting.

germinating in low temperatures after three and one-half months so that further sample plantings were impractical. Seeds which had received no treatment, as well as those which were stratified at 10° C., yielded no seedlings. These results indicate that a period of dry storage shortens the necessary after-ripening period. The seeds also showed better germination after three months of dry storage so there is no evidence that dry storage injured the seeds.

Two hundred seeds each were planted in flats which were put in open,

mulched, or board-covered cold frames. The fresh seeds were planted in October and the dried ones were planted in January. Table VIII gives the results of these tests. It will be seen that for the October planting the

TABLE VIII  
GERMINATION OF SEEDS OF AMELANCHIER CANADENSIS PLANTED IN COLD FRAMES

Condition	Per cent seedling production following spring	
	Fresh seeds planted in October	Seeds dried 3 mos. and planted in January
Open	20.5	14.5
Mulched	11.5	37.5
Board cover	40.0	23.0

board-cover condition gave the optimum temperature for after-ripening and subsequent germination. However, when the seeds were not planted until the following January the mulched condition proved best. This is doubtless due to the fact that after-ripening takes place more rapidly in the mulched condition. Hence, if the seeds are mulched in October, the after-ripening is complete, the seeds germinate and are killed before the mulch is removed. The use of mulch to produce the proper temperature for quick after-ripening can be used effectively only in case the planting is done at such time that mulch can be removed before seedling production has progressed to any great extent. These seeds showed low vitality as compared with the *Pyrus* seeds described above.

*Rose seeds.* Crocker (9) has found that seeds of roses of the cold temperate zone, whether species or hybrids, germinate well only if they are after-ripened by stratification at a low temperature or planted in a seed bed at low temperature. For *Rosa rubiginosa* stratification at 5° C. gave good germination while 0°, 10° C., and higher temperatures were not effective. He also found that rose seeds would germinate at 5° C. after they had had sufficient time to after-ripen. Calvino (7) recently stated that the hybrid rose seeds she is handling in Italy germinate well without low temperature stratification. She speaks of germinating them out-of-doors or in a cold greenhouse in the fall and winter, but gives no record of the temperature. It is possible that her seed beds had sufficiently low temperature during the winter to give the effect of low temperature stratification.

Table IX shows the course of germination of 12 different collections and 9 different species of rose seeds when they are kept in moist granulated peat at 5° C. The seeds of *Rosa canina* II, *R. carolina* II, and *R. multiflora* were purchased from a seedsman while all the rest were furnished by Professor A. C. Fraser of Cornell University, Ithaca, New York. The names used are those given by Professor Fraser. The germination tests were started in January following the harvest.

TABLE IX

GERMINATION OF ROSA SPECIES AT 5° C. IN PEAT; ALL SEEDS WERE STERILIZED FOR 30 MIN. IN 0.5 PER CENT USPULUN

Species	No. of seeds used	Per cent germination after months								
		2	4	6	8	10	12	18	24	27
<i>R. sp. I*</i>	1000	2.3	42.0	63.3	69.3	71.9	73.1	73.9		
<i>R. sp. II*</i>	1000	6.5	44.7	67.5	73.1	75.6	76.4	76.6		
<i>R. canina I</i>	1000	0	0	0.6	6.3	10.5	19.6	22.0	44.3	59.4
<i>R. canina II</i>	1000	0	0	0	1.5	1.9			6.2	
<i>R. carolina I</i>	1000	0.6	44.4	55.9					57.0	
<i>R. carolina II</i>	500	0	0	4.8	28.6	47.0		55.0	79.2	
<i>R. fendleri</i>	1000	0	0.5	0.6	0.7	1.1	2.0		11.8	21.5
<i>R. helenae</i>	300	0	2.0	8.0	14.0	15.0	15.3			
<i>R. multiflora</i>	200	26.0	72.5							
<i>R. rubrifolia</i>	1000	0	0.1	0.3	2.8	7.3	9.8		10.1	
<i>R. rugosa</i>	500	0	21.4	35.2	49.0		50.2	52.2		
<i>R. setigera</i>	1000	2.6	27.5	39.0	47.1	52.7	53.2	53.4		

\* A species used by Prof. Fraser as seed parent in some of his crosses but as yet not identified.

An inspection of Table IX will show that all the species and collections required considerable time to complete their germination. Ten of the 12 lots showed germinations on or after the eighteenth month in the germinator. *Rosa multiflora* showed the most prompt germination of any, completing germination with 72.5 per cent in four months. A number of other tests on the seeds of this species have all given similar prompt germination. The two lots of *Rosa sp.*<sup>1</sup> and lot I of *R. carolina* come next, but even these show some germination after 18 and 24 months. Then follow *R. rugosa* and *R. setigera*.

Of all the species tested, *Rosa canina* is the slowest to germinate. Lot I gave 59.4 per cent as the final germination after 27 months. Most of the germination occurred after 18 months. Lot II showed poor germination but in this also the germination occurred mainly after 18 months. Recently Miss Florence Flemion, working in this laboratory, has found that *R. canina* seeds after-ripen and germinate much more quickly at 1° C. than at 5° C. or higher temperatures. The other lots and species of seeds reported in this table showed poor germination and call for no further comment.

Crocker (11) states that *Rosa multiflora* seeds kept in dry storage will give good seedling production the second spring after harvest. Table X shows the germinative capacity of 11 different lots and 9 different species of rose seeds after two to four years of dry storage. It is to be regretted that four of these lots were not tested for germination soon after harvest. Aside from *R. canina* II, which showed only 6.2 per cent germination soon after harvest and 0.2 per cent after three years of storage, *R. carolina* with

<sup>1</sup> A species used by Professor Fraser as seed parent in some of his crosses, but as yet not identified.

TABLE X

EFFECT OF DRY STORAGE ON GERMINATION OF ROSA SEEDS; GERMINATED IN MOIST GRANULATED PEAT AT 5° C.

Species	Germination of freshly harvested seeds		Germination after dry storage of seeds		
	Per cent germination	After months in germinator	Period of dry storage in years	Per cent germination	After months in germinator
<i>R. blanda</i>	—	—	3	32.8	6
<i>R. canina</i> I	59.4	27	4	37.0	5
<i>R. canina</i> II	6.2	22	3	0.2	27
<i>R. carolina</i>	79.2	22	3	7.2	19
<i>R. humilis</i>	—	—	3	7.0	19
<i>R. lucida</i>	—	—	3	33.6	19
<i>R. multiflora</i>	72.5	4	3	48.4	5
<i>R. rubiginosa</i>	—	—	3	23.8	19
<i>R. rugosa</i> Thunb. (green)	49.5	10	3	53.0	19
<i>R. rugosa</i> (dried on bush)	27.2	11	3	51.0	13
<i>R. setigera</i>	53.4	18	2	35.6	15

79.2 per cent germination soon after harvest and 7.2 per cent after three years' storage, and *R. humilis* with no record after harvest and 7 per cent germination after three years of storage, the several lots of rose seeds have retained considerable germination capacity after two to four years' storage. It is of interest to note that *R. canina* I which showed 59.4 per cent germination just after harvest with 27 months in the germinator and only 0.6 per cent germination after six months in the germinator (Table IX) gave 37 per cent germination after five months for the three year old seeds. This indicates that these seeds partly after-ripen in dry storage. The degeneration of rose seeds in dry storage is relatively slow.

Many lots of hybrid rose seeds have been grown at this Institute for rose breeders in various parts of the United States. The method that has proved most effective is as follows: The seeds are removed from the hips, thoroughly cleaned and washed, mixed with granulated peat of proper moisture content, and placed in a 5° C. incubator or in an ice chest running between 5° and 10° C. Every week the seeds are examined, moldy seeds cleaned and sterilized, germinated seed pricked into small pots, and water added if necessary. The frequent examination is necessary in order to remove the seedlings before they become too big to be pricked into the soil.

The largest lot of hybrid rose seeds yet handled was the 1929 crop sent by Father George Schoener of Santa Barbara, California. This crop consisted of 357 different crosses with a total of 49,364 sinking seeds. Up to July 6, 1931, 18,358 seeds have germinated, giving 37.1 per cent germination. Between January 5, 1931 and July 6, 1931, 2511 seeds have germinated and several of the lots are still showing some germination at each examination. Up to July 6, 1931, there were still 41 crosses totalling 718

sinking seeds that had not yet begun to germinate. A considerable percentage of germinating seeds do not produce seedlings because of defective roots or weak plants that can not survive "damping off" organisms, mildew, etc. Somewhat less than 30 per cent of the germinated seeds die from these causes.

Father Schoener's 1930 crop of hybrid rose seeds is being handled in the same manner. This crop consists of 377 different crosses with a total of 29,451 sinking seeds. Up to July 6, 1931, 5103 seeds or 17.3 per cent have germinated.

Father Schoener's 1927 and 1928 crops of hybrid rose seeds, consisting of about the same number of seeds as the 1929 and 1930 crops, were handled by the ordinary methods used by rose breeders without the production of any seedlings. While the method here described may be defective in many respects, it is at least a great improvement over the methods ordinarily used.

In the light of the facts brought out in the experiments reported above for rose seeds and in the light of additional facts to be reported it is well to consider again the results reported by Calvino. The mean monthly temperatures at San Remo were not obtainable but at Nice, France, on the coast about 25 miles west of San Remo, the mean monthly temperatures for the five colder months of the year are as follows: November 50°, December 48°, January 46°, February 45°, and March 50° F. Recent unpublished work here at the Institute has shown that 15° C. is a very favorable temperature for the stratification of certain more southerly forms such as *Magnolia virginiana* L. and *Diospyros virginiana* L. Barton (5) has shown that 15° C., as well as 0°, 5°, and 10° C., are favorable temperatures for stratification of three of the four southern pines of the United States, *Pinus echinata*, *P. caribaea*, and *P. taeda*, while the northern pines (6) generally after-ripen best at temperatures below 10° C. In short, the more southerly temperate zone forms seem to respond better to higher stratification temperatures than the northern forms. Hence, San Remo winter temperatures may be near the optimum for after-ripening of Italian rose seeds. Even many of the northern temperate zone roses would probably find the five winter months at San Remo favorable for after-ripening and germination. This is true of the species *Rosa multiflora*, *R. rugosa*, and *R. carolina*. It is also true of many of the hybrids we have handled during the last five years. These favorable winter temperatures of Italy apparently furnish proper conditions for after-ripening Italian seeds and eliminate the necessity of stratification.

#### SUMMARY

The experiments in this paper deal with rosaceous seeds and cover two main points: A. Effect of temperature on after-ripening and germination; and B. Retention of vitality in dry storage.

A. Effect of temperature on the after-ripening and germination:

1. Apples ranging from one-half to two and one-half years old after-ripen and germinate well in three and one-half months at  $1^{\circ}$  and  $5^{\circ}$  C., and in an ice chest fluctuating between  $5^{\circ}$  and  $10^{\circ}$  C. A germinator at  $10^{\circ}$  C. is far less effective. The same seeds, when stratified for one month at  $1^{\circ}$ ,  $5^{\circ}$ , and at  $5^{\circ}$  to  $10^{\circ}$  C., then planted in a cold frame on March 15, give rather poor seedling production and when stratified at  $10^{\circ}$  C. for the same time and planted in the same way, give still poorer results. The same seeds stratified for two and one-half months followed by planting in a cold frame on May 1 give good seedling production for the three favorable stratification temperatures and poor seedling production for  $10^{\circ}$  C. stratification. Acid granulated peat (pH 4) and neutral granulated peat are equally effective as stratification media.

2. Apple seeds collected from pomace show poorer and slower germination than apple seeds taken directly from the apple. Either the cutting and pressing or the fermentation in the pomace causes a degeneration in the seeds.

3. Apple seeds after-ripen in the apple during cold storage. Such seeds retain their after-ripened condition even after a month of dry storage.

4. Peach seeds after-ripen and germinate well in three and one-half months at  $5^{\circ}$  and  $10^{\circ}$  C., but  $1^{\circ}$  C. is a less favorable temperature. Removal of the pericarp increases the per cent of germination. Seeds of Elberta peach with pericarps intact give no germination in a  $70^{\circ}$  F. greenhouse in three and one-half months but give a small germination per cent with pericarps removed. Lemon Free peach seeds give a low percentage germination at  $70^{\circ}$  F. in three and one-half months either with the pericarps removed or intact.

5. *Pyrus arbutifolia* seeds give good germination when stratified at  $1^{\circ}$  C. for two, three, or four months or at  $5^{\circ}$  C. for three months and then planted in a  $70^{\circ}$  F. greenhouse. Ten degrees C. gives little after-ripening of these seeds even after four months. Acid and neutral granulated peat are equally effective as stratification media.

6. *Pyrus arbutifolia* var. *atropurpurea* seeds after-ripen perfectly at  $1^{\circ}$  C. and at  $10^{\circ}$  C. in two months and give a big yield of seedlings when planted in a warm greenhouse. Neither *P. arbutifolia* nor the var. *atropurpurea* gives any seedlings when planted in a warm greenhouse without previous moist cold treatment.

7. *Amelanchier canadensis* seeds after-ripen well in four months at  $1^{\circ}$  C. and  $5^{\circ}$  C. Three and five months are less favorable stratification times. There is no after-ripening at  $10^{\circ}$  C., and seeds give no seedlings when sown in a  $70^{\circ}$  F. greenhouse without previous cold treatment. These seeds give good seedling production when planted in October in a cold frame pro-

tected with a board cover or when planted in a cold frame in January and protected with mulch and a board cover that holds the temperature of the bed a little above the freezing point.

8. It is shown for nine different species of rose seeds that they will after-ripen and germinate at 5° C. For *Rosa multiflora* the germination is complete in four months and for *R. canina* the germination is complete only after 27 months. The other species fall between these in speed of germination.

9. Work on hybrid rose seeds with several hundred different crosses and many thousand different seeds shows that plants can be produced effectively by placing the seeds in moist granulated peat at a constant temperature of 5° C. or a fluctuating temperature of 5° to 10° C., picking the seeds out as they germinate, and pricking them into pots.

#### B. Dry storage:

1. Seeds of the following apples, Patten Greening, Wealthy, and *Pyrus baccata* hybrid, taken from apples and stored dry for two and one-half years, germinate almost as well and give nearly as high seedling production as one and one-half year old seeds of the same sort stored similarly. Both of these show considerably lower germination than one-half year old seeds. Apple seeds fall in vitality relatively slowly and two and a half year old seeds are capable of producing a perfect stand if they are properly after-ripened previous to planting. Apple seeds that are to be used to produce understock or hybrid apple seeds will keep well if they are removed from the apples in the fall, cleaned, dried, and stored until time to stratify in late winter for spring planting.

2. Seeds of *Amelanchier* after dry storage for three months after-ripen more quickly and germinate better than freshly harvested seeds.

3. Both green and ripe seeds of *Rosa rugosa* show better germination after three years of dry storage than they do when fresh. *R. multiflora* gives 48.4 per cent germination after three years of dry storage against 72.5 per cent when fresh. *R. setigera* gives 53.4 per cent when fresh and 35.6 per cent after two years of dry storage. These and the other results reported in the table indicate that seeds of roses degenerate rather slowly in dry storage, retaining considerable germinating capacity after three years of storage.

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## SEASONAL DISTRIBUTION OF REDUCASE IN THE VARIOUS ORGANS OF AN APPLE TREE

SOPHIA H. ECKERSON

In the fall of 1925 the nitrate-reducing activity (reducase) was determined on samples of apple tree organs from the November 18 and December 18 collections made by Kraybill, Sullivan, and Miller (2) for their study of seasonal changes in the composition of Stayman apple trees. Reducase was relatively high in the fine roots and low in the above-ground parts. The buds had very little and the bark from one-year and two-year twigs had little more than a trace. Neither bark nor ground-up wood from the trunk, from the older branches, nor from the larger roots had any reducase. These facts seemed to indicate that if nitrate was being assimilated at that time it was reduced chiefly in the fine roots. This was in accord with Thomas' (5) conclusion, on the basis of his study of nitrogen metabolism throughout the year, that in apple trees amino acid synthesis takes place chiefly in the roots. But these reducase determinations were made at a season when there was little growth in any part of the tree. It was important to know the relative activity in the fine roots during the seasons of greater growth. In order to obtain that information this series of weekly determinations of reducase activity in the different organs of a single tree throughout the year was planned. Through the cooperation of the New Jersey Experiment Station, especially of Dr. G. T. Nightingale, the material for the study was obtained from a fruitful apple tree (*Pyrus malus* L. var. Stark) every week (except one) from March 31, 1930 to April 14, 1931.

During most of the year the ability of the tree to reduce nitrate was located chiefly in the fine roots. The principal exception was in the early spring when the buds were swelling. At that time reducase was high in both buds and roots but highest in the buds. That is the one time of the year when the buds as well as the roots contain nitrate.

### METHOD

The tree was given an application of nitrate April 11, 1930, and was watered when necessary during that unusually dry summer.

The samples were collected early in the morning and immediately wrapped in wet filter paper. Thus they arrived at the laboratory in turgid condition. This is important, since partial drying of the material greatly decreases the amount of reducase which can be extracted.

The roots were washed carefully and quickly patted between folds of filter paper to remove the adhering water. Then each sample was weighed, ground fine, and put in a beaker with a definite amount of water for each

gram of fresh material (usually 2.5 cc.) and mixed thoroughly. Toluene was added and the covered beakers were put in a refrigeration chest at about  $10^{\circ}$  C. After 24 to 30 hours the water extract was separated from the ground-up tissue by squeezing through four-fold cheesecloth. The nitrate-reducing power of these extracts was then determined in the manner described for plant juices, in an earlier paper (1). The residue was also tested to be sure that all the reducase had been extracted. The only tissue which sometimes retained a trace after extraction was that of the fine roots.

The reliability of the figures found for reduction of nitrate by an amount of extract corresponding to one gram of fresh material was checked

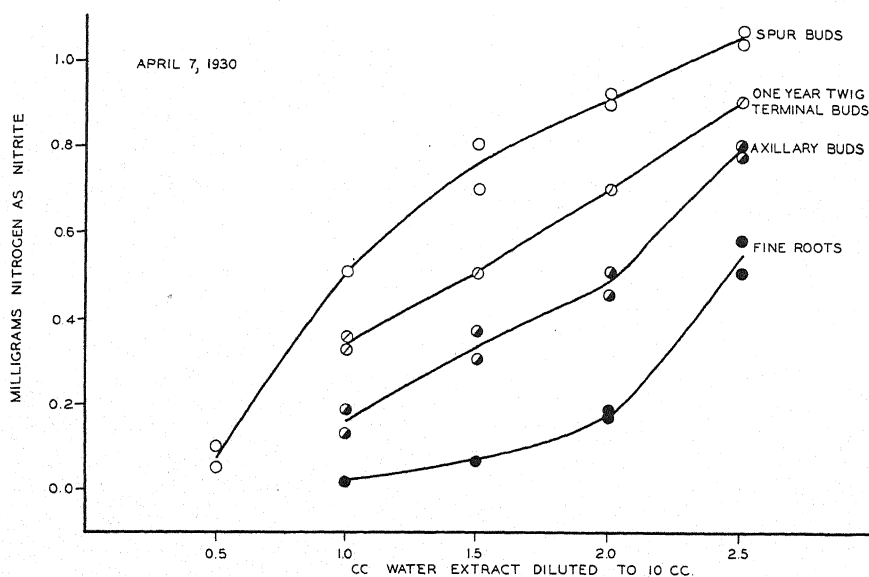


FIGURE 1. Reduction of nitrate to nitrite by different amounts of water extracts, from organs from a Stark apple tree, diluted to 10 cc. Notice that the amount of reduction is roughly proportional to the amount of extract and that the relative values for the different organs are of the same order for amounts of extract from 1 to 2.5 cc. Reduction by 2.5 cc. representing 1 gram of the fresh weight is taken as a measure of reducase activity.

each week by determining reduction by 10 cc. of solution containing different amounts of extract. One such set of determinations is shown in Figure 1. It can be seen that the reduction of nitrate is roughly proportional to the amount of extract and that reduction by 2.5 cc., corresponding to one gram of fresh material, represents very well the relative reducase activity of the different organs. The graphs shown in Figures 2, 3, and 4 are plotted on this basis.

## DISTRIBUTION OF REDUCASE

Reducase in the Stark apple tree was highest in the early spring from the time the buds began to swell until they were open. At this season reducase was high in the buds as well as in the fine roots (Fig. 2, March 31, April 7). The order of reducase activity of the buds from the different positions was: spur buds, terminal buds on one-year twigs, axillary buds. This was also the order of unfolding of the buds. April 7 most of the spur buds but only a few of the terminal buds were partly open, while the most advanced of the axillary buds were just showing green. A week later all of the spur buds and most of the terminal buds had opened out, while only a few of the axillary buds were open. At the same time the reducase had decreased greatly (April 14, Fig. 2). The amount in the spur buds was about one-tenth, and in the axillary buds one-fifth that of the previous week.

Reducase also decreased in the fine roots and since the new leaves had little, the reducase activity in the tree as a whole had decreased markedly. For the next three weeks growth of leaves and flower clusters was very rapid. Reducase in the leaves remained low and there was some in the flower cluster bases. When the flower buds were small (April 21) and when they were about to burst open (April 28) they contained a little reducase. The next week (May 5) pistils and stamens, peduncles, and the new stem were high in reducase (Fig. 2, legend).

Then came a period of extremely low reducase activity. And from May 19 to June 16 there was no reduction of nitrate by either extracts or juices from any of the organs. For comparison with the results from this particular tree, on June 9, Dr. Nightingale collected samples from a high-carbohydrate Stark apple tree growing in another county. But there was no nitrate reduction by extracts from any of the organs.

Reducase again appeared in leaves and fine roots June 23, and was found in the fruits for the first time. It remained low during July, except for a temporary rise in the leaves July 29 (Fig. 2). Notwithstanding the fact that the ability of the tree to reduce nitrate had been extremely low for several weeks, there was no lack of amides and amino acids for the developing seeds. June 30 and July 7 when sections of the seeds, which were still in the soft stage, were put in absolute alcohol the cotyledons became filled with many large crystals of asparagin and fewer smaller crystals of alanine, leucine, cystine, and other amino acids. No reducase was found in the seeds at any time during development.

Throughout the autumn reducase was fairly high in the fine roots and low in all other organs (Fig. 3). From the middle of August to leaf fall there was rarely more than a trace of reducase in the leaves. Through the autumn, beginning September 9, there was a little reducase in the bark of twigs of the current year's growth. After leaf fall there was a little reducase

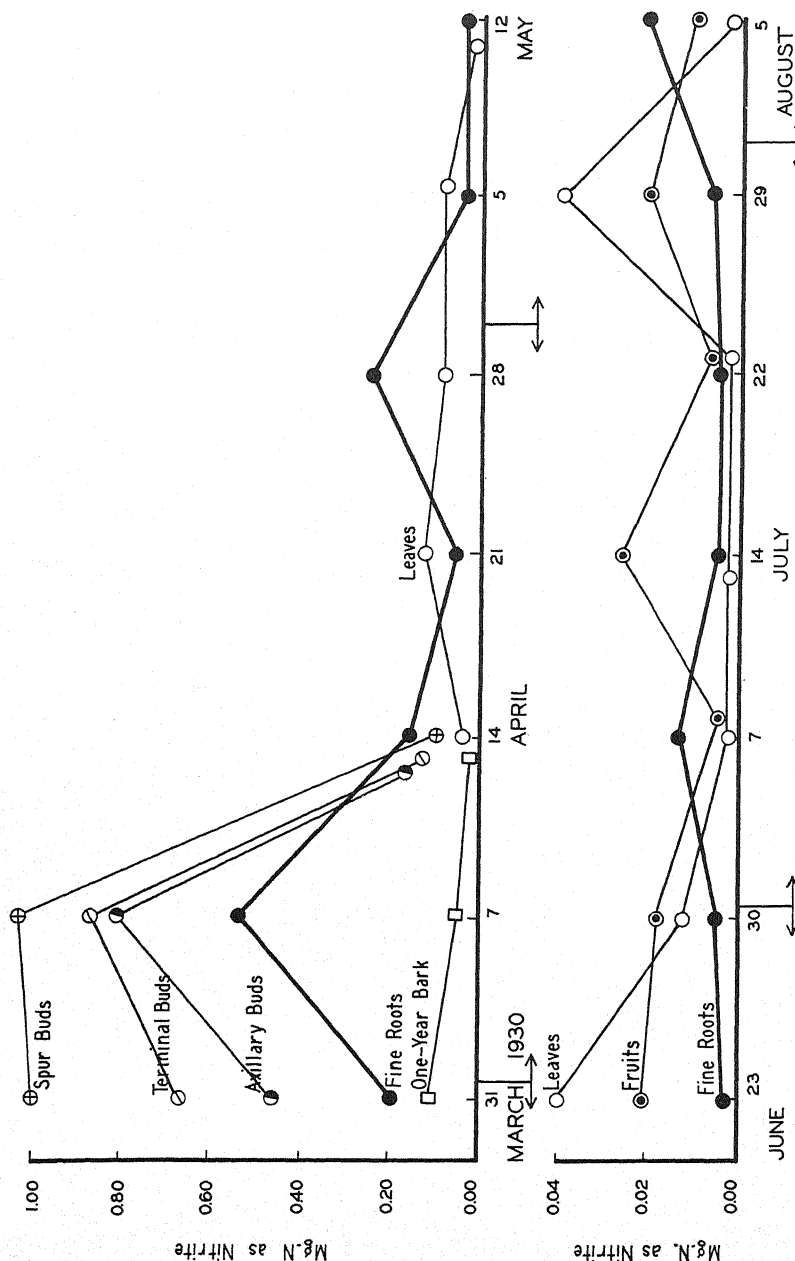


FIGURE 2. Reduction of nitrate by apple tree extracts from March 31, 1930 to August 5. At petal drop, May 5, pistil, peduncle, and new stem extracts reduced nitrate: 0.48, 0.30, and 0.24 mg. N as nitrite per gm. fresh weight. May 19 to June 16 there was no nitrate reduction by any of the organs. A high-carbohydrate tree also tested (June 9) gave no reduction.

in the buds. The buds were not tested before leaf fall, but it seems possible that there might have been some as early as September 16, since it appeared in the bark at that time. It was found throughout the autumn and

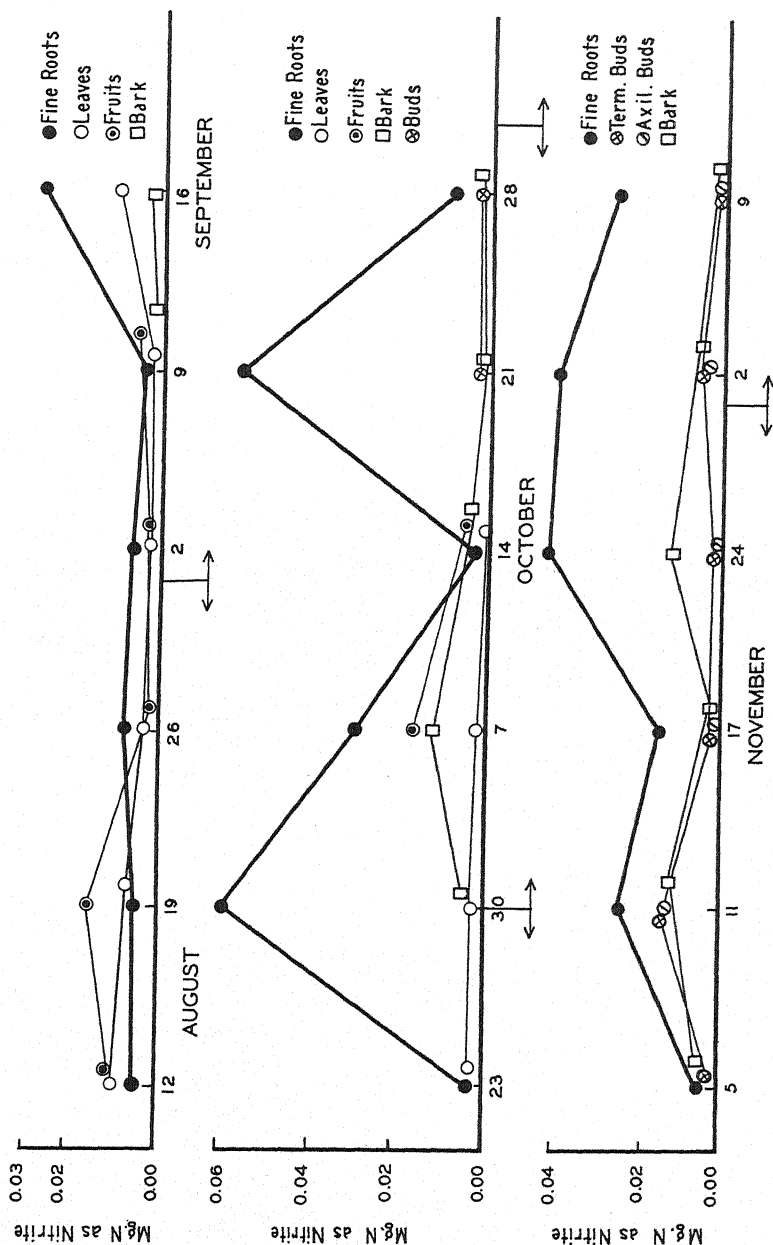


FIGURE 3. Reduction of nitrate by apple tree extracts from August 12 to December 9. The leaves fell between October 14 and 21. Notice the similarity between reduction by bud and bark extracts.

winter that reducase activity in the young bark accompanied, or in early spring possibly preceded, that in the buds.

In December and early January reducase was high in the fine roots,

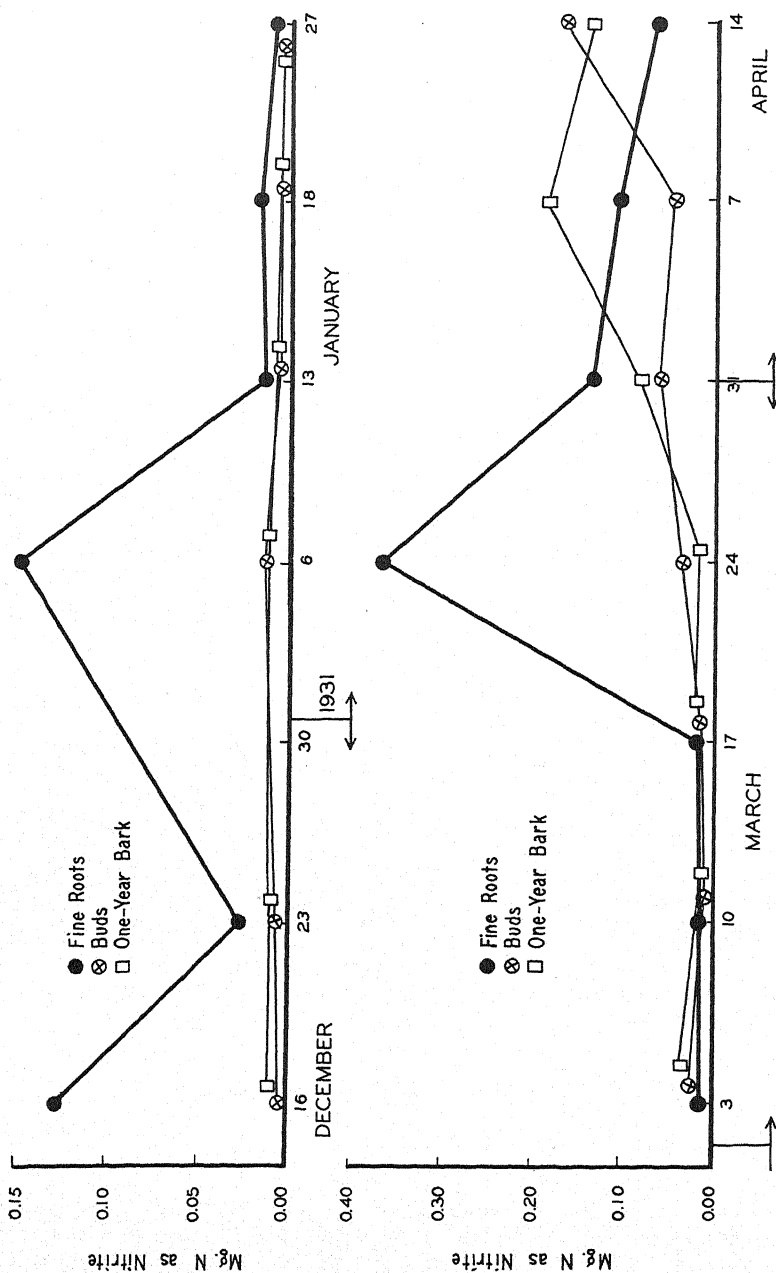


FIGURE 4. Reduction of nitrate by apple tree extracts from December 16 to April 14, 1931. From February 2 to 25, there was only a trace of nitrate reduction by any of the organs.

while there was very little in the buds and bark (Fig. 4). The reductase then decreased in the fine roots and during February there was only a trace found in roots, buds, or bark. In March the reductase in the fine roots rose

rapidly to a maximum, then dropped a little as it increased in the buds and the bark. The terminal buds collected April 14 were just showing green.

#### DISCUSSION

In the early spring of 1930 the fine roots contained much nitrate, while the buds of March 31 and April 7 contained considerable. April 14 there was only a trace in the buds and after that none was found in the above-ground parts until the next spring. In 1931 the buds of March 31 and April 7 and 14 contained nitrate.

In the spring of 1922 and of 1923 buds from several apple trees in the University of Wisconsin orchard were examined. Nitrate was always found at the time the buds began to swell. It was not found in the young leaves as the buds opened. In later years buds from apple trees in other orchards were examined with similar results. Thomas (5) also found tests for nitrate "... negative in the aerial parts except in the buds as they were opening." It seems safe to conclude that if there is nitrate in the soil some will be carried to the buds when they begin to swell in the spring, and that for a short period the buds (and adjacent bark) take an active part in the reduction of nitrate and synthesis of amino acids.

Reducase was very low in the leaves throughout their cycle. No nitrate was found at any time. These facts indicate that in the apple tree the leaves can play but a small part in the reduction of nitrate and synthesis of amino acids. Their work seems to be rather the synthesis of carbohydrates.

Although apple leaves do not ordinarily contain nitrate, they may under some unusual condition. One example has been noted. Two trees in an apple orchard of the University of Wisconsin were heavily nitrated shortly after the flowers had opened. Within three days the lower leaves (at the five foot level) contained nitrate. But in about a week all nitrate had disappeared and no more was found in the leaves that season.

Reducase in the fine roots was low in summer, increasing in the fall. It was high in winter reaching a maximum in the early spring when it was also high in the buds.

If the amount of reducase in the extracts is an indication of the amount of nitrate reduction in the tree, then nitrogen in the tree should increase markedly from leaf fall to leaf appearance in the spring but should not increase during the summer. This seems to be in accordance with Sullivan and Kraybill (3), especially with their midwinter and summer findings. They find, between December 18 and February 26, an increase in all forms of nitrogen in all parts of the tree, and from April through July a decrease in total nitrogen on the dry weight basis.

During the fall and winter until early January developmental changes in the fruit buds could be seen from week to week. Growth in the bud interior slowed during December but did not cease until in January. The



protoplasm of buds collected January 6 still appeared translucent, but in those of January 13 it appeared opaque. The fine roots remained active a little later than the buds, and became active again a little earlier than the buds (fruit buds and terminal vegetative buds). This conclusion is based on the appearance of the protoplasm of the parenchyma cells back of the tip, and on apparent chemical changes taking place in the slightly older parts as shown in microchemical examination. The difference in the appearance of protoplasm in dormant compared with active tissue has been noted by Swarbrick (4). He describes the change in appearance of the cambium of apple trees from "dirty grey" in winter to "swollen and translucent" in spring.

No doubt Sullivan and Kraybill (3) are right in the suggestion that their apple trees in eastern Pennsylvania continued absorption and synthesis of nitrate between December and February. At least the roots of this Stark apple tree in northern New Jersey did not become dormant until late January.

#### SUMMARY

1. The reducase activity of a Stark apple tree was determined at weekly intervals from March 31, 1930 to April 14, 1931.
2. The ability of this apple tree to reduce nitrate was highest in early spring when the buds were swelling and lowest (zero) during the five weeks following flowering; it was low during summer, increasing in late summer and early fall; it was high from late fall to late winter, dropping to extremely low during February; then there was a rapid rise to a maximum in early spring.
3. The high reducase during fall and winter was localized in the fine roots. The maximum reducase in early spring was localized in both fine roots and buds.
4. Reduase in the buds was always accompanied by reducase in the adjacent bark.
5. There was very little reducase in the leaves at any time.

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# AFTER-RIPENING, GERMINATION, AND VITALITY OF SEEDS OF *SORBUS AUCUPARIA* L.

FLORENCE FLEMION

## INTRODUCTION

The seeds of many rosaceous plants are dormant at maturity, that is, they will not germinate readily when put under conditions favorable for germination, but have to undergo some changes usually spoken of as after-ripening before germination can occur. The mechanism by which such dormancy is secured in seeds has been described by Crocker (1) and he emphasizes the necessity of considering both the embryo and the seed coat in any dormancy problem.

The seeds of *Sorbus aucuparia* L. (*Pyrus aucuparia* (L.) Ehrh.) commonly known as European mountain ash, which belongs to the Rosaceae family, offer excellent material for a study of the factors connected with dormancy as the seed coats can be removed without injury to the well matured embryo. In addition the seeds have very thin endosperm so the excised embryos suffer little loss of storage material, since most of the food materials are stored in the cotyledons. It is, therefore, possible to study the dormancy of the embryos as well as the dormancy of the intact seeds. In the case of many species which have dormant seeds this cannot be done because such factors as hard seed coats, large endosperm, partially developed embryos, etc., make it either difficult to excise the embryos without injury or difficult for the excised embryos to survive the loss of much or all of their storage food.

It was thought desirable to make an extensive study of the factors connected with the dormancy of this seed in the hope that some of the facts learned might be applied to other dormant seeds as well. It seemed that perhaps more progress could be made by studying one kind of seed very thoroughly rather than by making the various tests on many kinds of seeds. In this paper are reported the results of extensive experiments on the methods of breaking dormancy such as effects of low temperature stratification, chemical treatments, condition of storage, and various other agents. The factors involved in dormancy were also studied by tests on the excised embryos and by studying the enzyme changes taking place while the intact seeds were undergoing after-ripening.

## MATERIALS

The berries, except where otherwise indicated, were collected when mature in early fall (September or October) on the Samuel Untermeyer estate at Yonkers, New York. Immediately after collection, the seeds were freed from the pulp by the use of a Hobart mixer, washed well, spread out in thin

layers for thorough drying, and stored in open containers at room temperature. In certain cases as specifically noted below, the berries were purchased from seedsmen and obtained as soon after harvest as possible. They were then cleaned, dried, and stored as described above. In every case, the seeds collected at Yonkers gave an earlier and a higher percentage germination than those received from seedsmen. It may be that the development of heat in fermenting berries stored in bulk during shipment had some deleterious effect.

## BREAKING DORMANCY

### LOW TEMPERATURE EFFECTS

These seeds require a low temperature for the after-ripening which must precede germination. They not only after-ripen but also germinate at a low temperature.

#### *After-ripening and Germination at Low Temperatures*

When the seeds are stratified (that is, mixed with moist peat moss and placed in bottles) (3, p. 116) and kept in constant temperature ovens, germination takes place in two to four months at 1° C. (11), less readily in three to five months at 5° C., and only rarely at 10° C. or above. A fluctuating temperature of 2° to 8° C. (ice chest) as well as 1° to 5° C. alternated weekly or daily (18 hrs. at 1° C. and 6 hrs. at 5° C.) was also found to be extremely effective in after-ripening these seeds. Table I which shows per

TABLE I  
AFTER-RIPENING AND GERMINATION OF SEEDS OF *SORBUS AUCUPARIA*

Stratification temperature °C.	Per cent germination						
	54 days	68 days	82 days	99 days	113 days	127 days	152 days
1			3	70	93	94	
5	3	5.5	8	15	28.5	39	
10							0.5
15						0.5	
Room temp.							0

cent germination obtained with seeds while in stratification at various temperatures is typical of results obtained. (The germination data in all the tables presented in this paper, unless otherwise indicated, were obtained from lots of 200 seeds each.) It is seen that germination at 5° C. started earlier than at 1° C., but the germination at 1° C. soon overtook that at 5° C. so that the total germination was obtained much earlier at 1° C. Although after 82 days there was 8 per cent germination at 5° C. and only 3 per cent at 1° C., after 99 days 70 per cent had germinated at 1° C., 39 per cent at 5° C., and only 0.5 per cent at 10° C. The results, when seeds had

been stratified three months at  $1^{\circ}$ ,  $5^{\circ}$ , and  $10^{\circ}$  C., then planted in a greenhouse flat and photographed two months later, are illustrated in Figure 1. The stratification at  $1^{\circ}$  C. produced 74 per cent of fine seedlings, at  $5^{\circ}$  C. 11 per cent, and at  $10^{\circ}$  C. only 0.5 per cent. Seeds were stratified at  $-8^{\circ}$  C. for one, two, and three months and at  $-5^{\circ}$  to  $+5^{\circ}$  C. alternated weekly

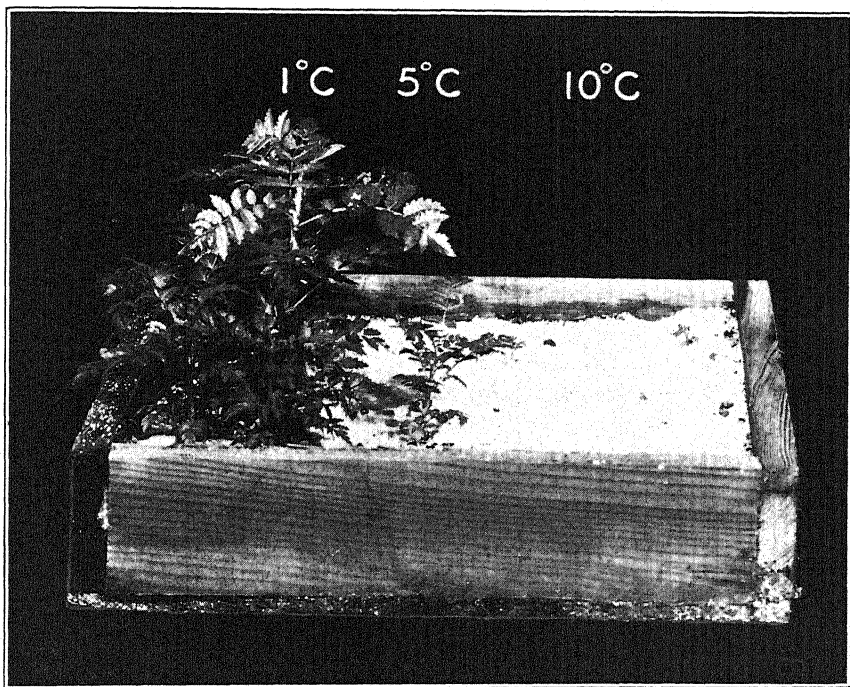


FIGURE 1. The effect of three months' stratification of *Sorbus aucuparia* seeds at temperatures of  $1^{\circ}$ ,  $5^{\circ}$ , and  $10^{\circ}$  C. upon subsequent seedling production when planted in greenhouse flats.

for two and three months. No germination resulted when these were then planted in greenhouse flats.

*Stratification medium.* When peat moss, sand, and muck were tested as media for stratification at  $1^{\circ}$  C. it was found that peat moss gave the best results. Since commercial granulated peat moss<sup>1</sup> is always acid in reaction, pH about 4, experiments were run to determine whether peat moss adjusted to other pH values would give better results. The peat was made more alkaline by adding various amounts of calcium carbonate as described by Hitchcock (14, p. 136) and made more acid by adding various

<sup>1</sup> Granulated peat moss was obtained from Atkins and Durbrow, New York City.

amounts of hydrochloric acid. For pH determinations the quinhydrone electrode was used. The results are shown in Table II. The pH values are

TABLE II  
PER CENT GERMINATION OF SEEDS STRATIFIED AT 1° C. IN PEAT MOSS AT VARIOUS  
HYDROGEN ION CONCENTRATIONS

Treatment per liter of peat moss	pH values of medium			Per cent germination				
	At beginning of experiment	After six weeks	After nine weeks	63 days	70 days	77 days	84 days	91 days
200 cc. N-HCl	2.15	2.17	3.16					
250 cc. N/10 HCl	2.88	2.79	2.49					
Check	4.11	4.14	4.11	24	50	70	79	84
1 g. CaCO <sub>3</sub>	4.88	4.85	4.98	12	30	54	69	79
3 " "	5.92	6.01	6.94	10	20	20	42	56
5 " "	7.70	6.79	7.53	2	5	18	25	45
10 " "	7.62	7.70	7.97	6	11	20	38	63
20 " "	7.78	7.82	7.95	6	14	21	40	53

given for the various lots at the beginning of stratification and after six and nine weeks. It is seen that untreated peat moss gave better results than any of the lots adjusted to other pH values, the more acid media being definitely injurious. In experiments not shown in the table, the medium of each lot was replaced after three and six weeks by untreated peat moss. When seeds were kept in the more acid media for only three or six weeks the injury was only slight as compared with the considerable injury resulting when seeds were kept in these media for the whole stratification period. That is, when the seeds were kept in an acid medium of pH 2.88 for three weeks then transferred to untreated peat moss 64 per cent germinated in 91 days, and when kept in acid medium for six weeks 60 per cent germinated in 91 days, while if kept in the acid medium for the whole period none germinated. Apparently there was an almost complete recovery from the acid effects when the seeds were kept in the acid media only six weeks. Such a difference was not found for the more alkaline media. The germination of the seeds when kept in the more alkaline media for three and six weeks was only slightly higher than the seeds kept in this media for the whole period. Of all the media tested for stratification at 1° C., untreated peat moss gave the best results.

### *Cold Frames*

When seeds were planted out-of-doors in cold frames under three conditions, in cold frames without cover, in cold frames with a board cover, and in cold frames within which the seeds were mulched (covered with leaves) and then covered with a board cover, the best seedling production was obtained in the second condition where the seeds were kept in cold

frames with just a board cover. On January 5, 1929, flats with 500 seeds each of the 1928 crop were placed under the conditions indicated above and the germinations resulting the three following springs recorded. Results are shown in Table III together with results from flats which were placed inside in greenhouses kept at 21° C. (70° F.) and 5° to 10° C. (40° to 50° F.)

TABLE III  
SEEDLING PRODUCTION OF *SORBUS AUCUPARIA* WHEN SEEDS ARE PLANTED IN FLATS AND HELD IN DIFFERENT CONDITIONS

Location of flats	Per cent germination			
	4-6 mos. April-June 1929	7-10 mos. July-Oct. 1929	16-18 mos. April-June 1930	27-30 mos. April-June 1931
Greenhouse 21° C. (70° F.)	0.2	0.2	0.2	0.2
Greenhouse 5°-10° C. (40°-50° F.)	2.4	3.8	4.0	4.0
In mulched cold frame	21.8	21.8	26.8	26.8
In open cold frame	40.4	41.2	41.4	41.4
Board cover cold frame	71.4	71.4	73.2	73.2

respectively, at the same time. There was practically no germination in the case of the greenhouse flats. The first spring following the planting, 71.4 per cent had produced seedlings in the cold frames with the board cover, 40.4 per cent in the open frames, and 21.8 per cent in the case where the seeds were mulched and the cold frames covered. The percentage of seedlings obtained was not appreciably increased the second spring and no further seedling production resulted the third spring.

In this connection it should be noted that as described by Crocker (3, p. 119) certain cold frame conditions may not give good seedling production because many of the seeds may germinate during the winter and perish under the mulch. Thus, with proper mulching, a temperature around the freezing point is obtained and since this is the correct temperature for the after-ripening and germination of *Sorbus aucuparia* seeds, seedlings are probably produced during the winter and die under the mulch. This is especially likely in this experiment for the favorable condition was maintained four months before the mulch was removed while germination begins after two months. Under the board cover the temperature is below freezing much of the time so that in four months under this condition the favorable after-ripening temperature of 1° C. is maintained for only part of this time, and the seeds do not germinate to any extent during the winter but nevertheless after-ripen sufficiently for germination to take place in the spring. This may be the reason why better results were obtained when the seeds were planted in cold frames with just a board cover. If the seeds were mulched at the proper time during the winter so that they would be subjected to a low temperature for a sufficient length of time to after-ripen and

still not germinate until spring, a higher percentage of seedlings might be obtained. This, however, is not very practical since the ground is frozen and it would be necessary to plant the seeds in flats and then place them in cold frames.

In another experiment, seeds were planted out-of-doors in cold frames under the conditions described above in October and December 1929, and February and April 1930. Best results were again obtained in the cold frames with a board cover while the open frames gave the poorest seedling production and the results obtained with the mulched conditions were intermediate between those of the open frames and the frames with a board cover. Those planted in October and December under a board cover gave a high percentage of seedlings in the spring of 1930; those planted in February under the same conditions gave a low percentage the following spring and a higher percentage the second spring (1931); while those planted in April and kept under a board cover did not germinate until a year later.

### *Seedling Production*

When the seeds of *Sorbus aucuparia* are planted out-of-doors in the temperate zone in the fall or early winter, germination follows in the spring; when planted in the spring or summer, none germinate until the following spring. When the seeds are stratified in moist peat moss at 1° C., they germinate in two to four months while in stratification. The method suggested for the optimum production of seedlings is to take seeds which have been properly obtained from the berries and stored dry at room temperature as described elsewhere in this paper and stratify them in moist peat moss for a period of two to four months at 1° C. or until seedlings are noticed in the stratification medium, and then plant outside or in the greenhouse, as the season permits. If the seeds are to be planted out-of-doors, the time when the stratification period is begun must be so adjusted that the seedlings will not be produced at a time when the outside temperature is so low as to freeze the seedlings. That is, if the plantings are to be made on April 15, stratify on February 1, except when the seeds are obtained from a seedsman, stratification should begin about a month earlier. If it is not possible to use low temperature stratification, a fair seedling production will be obtained by planting the seeds in late fall out-of-doors in cold frames with a board cover.

### CHEMICAL TREATMENTS

Many attempts were made to shorten or replace the required low temperature stratification period by treatments with chemicals. In addition to ethylene chlorhydrin, thiourea, and thiocyanates which were found by Denny (7) to be very effective in breaking the dormancy of potato tubers, many other chemicals were tried. When the chemicals were soluble in wa-

ter, solutions of various concentrations were made up and the seeds soaked in these solutions for periods of from one to seven days, usually at ice box temperature, and subsequently stratified at 1° C. In the case of some of the chemicals, seeds were exposed to vapors of the chemical for various periods.

Some of the chemicals tested in addition to those mentioned above were potassium cyanide, urea, saponin, vanadium compounds, triethanolamine, organic and inorganic acids, alcohols, aldehydes, glycerine, hydrogen peroxide, adrenalin, and cyanates. It is not practical to give in this paper a complete list of all the conditions of treatment tried with these various chemicals. Usually about five or six concentrations were tested, varying from 5 per cent to 0.005 per cent. With many chemicals a wider range of concentration was tried and in addition the time and temperature of the treatment varied.

Attempts were also made to shorten the rest period by soaking the seeds for various lengths of time in seedling extracts.

In no case was any evidence obtained of any appreciable shortening of the low temperature after-ripening period required. With many chemicals the high concentrations tolerated indicate that in all probability many substances did not penetrate into the seed. On the other hand some chemicals gave evidence of injury showing that the seed coats were permeable to these chemicals at least.

In all these tests two controls were used, one being soaked in water for the same length of time as used for the chemical treatments and the other being an untreated control. Soaking in water always had a slight effect in hastening germination, with usually about 20 per cent more germination after 75 days of stratification than the seeds which were dry when stratified.

It must not be concluded from these results, however, that no chemical can be found which will be effective in breaking the dormancy of these seeds. Recently Deuber (9) has reported a beneficial effect of ethylene chlorhydrin and thiourea on seeds of the sugar and Norway maples and on acorns of the black and red oaks. It has not been possible to obtain results with these chemicals in *Sorbus aucuparia* seeds. It should be noted, however, that the seeds of *Sorbus aucuparia* are more dormant than the seeds used by Deuber.

#### MISCELLANEOUS METHODS

Various other conditions and methods were tested as to their effect on the dormancy of these seeds. Seeds were kept at 1° C. under germination conditions in atmospheres containing various amounts of oxygen up to 80 per cent. Also seeds were placed in water and subjected to hydraulic pressure up to five tons per square inch for various lengths of time. Attempts were made to introduce water into the seeds by placing them in water and



putting them under a vacuum and then releasing the vacuum, the process being repeated several times.

The effect of exposing the seeds to ultra violet light for various periods and subjecting them to electro-magnetic waves was also studied. None of these treatments resulted in any effect, the germination being neither hastened nor retarded under the conditions of these experiments.

#### VITALITY AND AFTER-RIPENING IN STORAGE

When seeds have been in dry storage for six months in room temperature and are then germinated at 1° C. there is a decrease in the length of the stratification period required for germination. Table IV shows the per cent germination at 1° C. of freshly harvested seeds and of seeds of the same

TABLE IV  
PER CENT GERMINATION AT 1° C. OF *SORBUS AUCUPARIA* SEEDS IN DRY  
STORAGE FOR VARIOUS PERIODS

Length of storage period	Per cent germination after stratification for various numbers of days												
	40	48	54	61	68	75	82	88	99	106	113	120	127
Freshly harvested						0.5	3	12	70	87	93	93.5	94
Dry stored 3 months				5	26	50.5	73	81.5	90				
Dry stored 6 months	1.5	14	46	68.5	90.5	92	95.5						
Dry stored 12 months			4.5	19.5	52	67.5	75.5	77.5	78.5	81.5	85	87	

lot after 3, 6, and 12 months' dry storage at room temperature. The freshly harvested seeds began their germination in 75 days with 0.5 per cent, those dry stored three months in 61 days with 5 per cent, those dry stored six months in 40 days with 1.5 per cent, while those dry stored twelve months in 54 days with 4.5 per cent. The germination was completed by freshly harvested seeds in 127 days, by those stored three months in 99 days, by those stored six months in 82 days, and by those stored twelve months in 120 days. It is seen that the seeds which had been stored for six months required a shorter stratification period than those stored for shorter or longer periods. Similar results were obtained with other lots of seeds. Undoubtedly the seeds partially after-ripened in dry storage at room temperature. It appears that after storage for too long a time this effect is again lost. Experiments were undertaken with different conditions of storage of the seeds to determine whether any condition could be found which would shorten the stratification period to a greater extent than ordinary dry storage at room temperature for six months and also to determine the best storage condition for these seeds from the viewpoint of retention of vitality.

TABLE V  
THE EFFECT OF SEALED AND UNSEALED STORAGE OF AIR-DRY SEEDS AT VARIOUS TEMPERATURES FOR ONE YEAR UPON SUBSEQUENT PER CENT GERMINATION WHEN STRATIFIED AT 1° C.

Storage temperature ° C.	Per cent germination													
	1 month sealed	1 month unsealed	1½ months sealed	1½ months unsealed	2 months sealed	2 months unsealed	2½ months sealed	2½ months unsealed	3 months sealed	3 months unsealed	3½ months sealed	3½ months unsealed	4 months sealed	4 months unsealed
-8														
1		0.5		5	1	1	5	50.5	37	55.5	60.5	74	81.5	80.5
5		0.5		2	1	7	14.5	28	24.5	62	52.5	94	70	100
10				2	5	10	27.5	18	48	47.5	80	70.5	92	87
15	0.5				10	3	14.5	18	62.5	54	88.5	84	93.5	93
20				4	3.5	1.5	24	17	50.5	51	80.5	97	83	99.5
Room temperature					5	28		58	60.5	78	72.5	97	83	97
25						19.5	Mold	67.5		78		81.5		81.5

*Air Dry Seeds Stored in Various Conditions*

Lots of 200 seeds each were kept sealed (in 50 cc. bottles corked and sealed with DeKhotinsky cement), unsealed (in uncorked 50 cc. bottles), and exhausted (in 100 cc. Pyrex flasks exhausted with a vacuum pump and then sealed with a flame) for periods of 3, 6, 12, and 24 months, at temperatures of  $-8^{\circ}$ ,  $1^{\circ}$ ,  $5^{\circ}$ ,  $10^{\circ}$ ,  $15^{\circ}$ ,  $20^{\circ}$ ,  $25^{\circ}$  C., and at room temperature. The seeds were then stratified at  $1^{\circ}$  C. and the per cent germination recorded. Table V gives the germination record of seeds which had been kept sealed and unsealed at these temperatures for one year and Table VI shows the germination record of seeds which had been kept under vacuum at various temperatures for two years as well as that of an unsealed room temperature control. The data here presented as well as the data for the seeds stored for the other periods of one and two years show no significant difference either in the total germination obtained or in the length of the stratification period required to reach this germination, except that storage at  $25^{\circ}$  C., under all of the conditions tested at this temperature (sealed and unsealed), resulted in the death of the seeds.

TABLE VI  
THE EFFECT OF STORAGE OF AIR-DRY SEEDS UNDER VACUUM AT VARIOUS TEMPERATURES FOR TWO YEARS UPON SUBSEQUENT GERMINATION WHEN MIXED WITH MOIST PEAT MOSS AT  $1^{\circ}$  C.

Storage temperature	Per cent germination				
	2 months	2½ months	3 months	3½ months	4 months
$-8^{\circ}$ C.	2	7.5	47.5	84.5	90
$1^{\circ}$ C.	4.5	17.5	55	87	92
Ice chest	6	22.5	70.5	88	92
Room temperature	3	9	54	82	86
Room temperature unsealed	1.5	20	48.5	68	77.5

These experiments show, therefore, that the seeds after two years of storage are just as viable as the freshly harvested seeds, whether stored sealed, unsealed, or in vacuum at temperatures from  $-8^{\circ}$  C. to room temperature. At  $25^{\circ}$  C. the vitality was rapidly lost. No condition was found under which the seeds after-ripen to a greater extent than at dry storage at room temperature for six months.

In another experiment, intact berries were stored unsealed at temperatures from  $-8^{\circ}$  C. to room temperature for one and two years, then cleaned, and stratified at  $1^{\circ}$  C. Stored cleaned seeds and the seeds from the intact berries stored at low temperatures gave much better germination percentages than the seeds from the intact berries stored at high temperatures.

*Storage at Various Relative Humidities*

Beginning in the fall of 1929 seeds were stored at room temperature for periods of one-half, one, and two years at various humidities maintained by keeping the seeds in desiccators over the required concentrations of sulphuric acid (20), the controls being kept in a desiccator containing nothing but the seeds. The desiccators were opened only when seeds were removed at the end of one-half, one, and two years. Table VII shows per cent ger-

TABLE VII

THE EFFECT OF ROOM TEMPERATURE STORAGE OF SEEDS AT VARIOUS HUMIDITIES FOR DIFFERENT LENGTHS OF TIME UPON SUBSEQUENT GERMINATION WHEN STRATIFIED IN PEAT AT 1° C.

Conditions under which stored in desiccators	Length of time stored in years	Per cent germination when stratified at 1° C.						
		1 month	1½ months	2 months	2½ months	3 months	3½ months	4 months
Conc. H <sub>2</sub> SO <sub>4</sub>	½ 1 2			0.5	3	4	Mold	Mold
Anhydrous CaCl <sub>2</sub>	½ 1			0.5 6.5	2 15	6 18.5	8 21.5	35
55.58% H <sub>2</sub> SO <sub>4</sub> (About 25% humidity)	½ 1 2	1	13 2	65.5 23 16.5	92.5 51 69	64 88	89 89	91 90
43.14% H <sub>2</sub> SO <sub>4</sub> (About 50% humidity)	½ 1 2			32.5 15.5	83.5 32	87.5 47 0.5	70 6.5	75 18
30.2% H <sub>2</sub> SO <sub>4</sub> (About 75% humidity)	½ 1 2				Mold	Mold		Mold
Control	freshly harvested ½ 1 2	0.5	14 3.5	68 24.5 6	1.3 92 46 57	21 95.5 72 81	82.5 85 85	94 87 89.5

mination of lots of 200 seeds each from these experiments when subsequently kept in moist peat moss at 1° C. The seeds stored over concentrated sulphuric acid were so severely injured that practically none germinated and those stored over anhydrous calcium chloride were also definitely injured. When stored in approximately 25 per cent relative humidity the results were not different from the controls, that is, 92 per cent germinated in two and one-half months after six months' storage and about 90 per cent germinated in four months after either one or two years' storage. In both the controls and those in about 25 per cent humidity the stratification period after one and two years' storage was about the same as that required by freshly harvested seeds, while those stored for six months required a

shorter stratification period. In the case of those stored in approximately 50 per cent relative humidity, 87.5 per cent germinated in three months after six months' storage, 75 per cent in four months after one year's storage, and 18 per cent in four months after two years' storage, the deleterious effect augmenting with the period of storage. Storage at 75 per cent relative humidity was even more injurious.

Seeds stored at room temperature at approximately 50 per cent and 75 per cent relative humidity were considerably injured, when over concentrated sulphuric acid or anhydrous calcium chloride they were still more injured, while those stored at approximately 25 per cent relative humidity were no different from the controls kept under the conditions prevailing at ordinary room temperature. Heinrich (13) has also shown that seeds have a critical moisture content below which the keeping quality is good and above which the keeping quality is poor. This critical moisture content is near or a little below the water content of thoroughly air dry seeds in our climate.

Other experiments were also carried on where lots of 200 seeds were stored at various relative humidities at various temperatures for 4, 6, 9, and 12 months and then placed in moist peat moss at 1° C. Table VIII shows the effect of storing seeds for four months at 1°, ice chest temperature (2° to 8° C.), 15° C., and room temperature in conditions ranging from approximately 90 per cent relative humidity down to the condition maintained by concentrated H<sub>2</sub>SO<sub>4</sub>. Similar results were obtained for the seeds stored 6, 9, and 12 months. It is readily seen that the seeds stored at 1° C. in all the conditions tested gave good germination, except that those stored at 90 per cent humidity and those over concentrated sulphuric acid gave evidences of some injury. When stored in the ice chest and at 15° C., the seeds in 25 per cent, 50 per cent, and 75 per cent humidity, and the controls gave a good per cent germination; while those stored over concentrated sulphuric acid or over calcium oxide showed a very slight injury in the ice chest and a very decided injury at 15° C. When stored at room temperature, the seeds in 10 per cent, 35 per cent, and 50 per cent humidity, and the controls gave a good per cent germination; while those in 65 per cent humidity and 75 per cent humidity and those over concentrated sulphuric acid or over calcium oxide gave evidence of much injury. At the low temperature of 1° C., the seeds survived all the conditions tested. At room temperature, the seeds were injured when stored over concentrated sulphuric acid or calcium oxide or when stored at humidities above 50 per cent.

The variation in relative humidity which these seeds can tolerate in 12 months' storage and still retain their vitality decreases as the temperature rises. Other workers (13, 16) have shown that the water content may be increased without injury to the seeds in storage if the temperature is

TABLE VIII  
THE EFFECT OF STORAGE OF SEEDS FOR 4 MONTHS AT VARIOUS TEMPERATURES AND HUMIDITIES UPON THE SUBSEQUENT STRATIFICATION PERIOD REQUIRED

Storage temperature °C.	Germination period at 1° C. in months	Per cent germination in 1, 2, 3, and 4 months at 1° C. after 4 months storage at various humidities and temperatures									
		Conc. H <sub>2</sub> SO <sub>4</sub>	CaO	64% H <sub>2</sub> SO <sub>4</sub> (About 10% humid.)	55% H <sub>2</sub> SO <sub>4</sub> (About 25% humid.)	50% H <sub>2</sub> SO <sub>4</sub> (About 35% humid.)	43% H <sub>2</sub> SO <sub>4</sub> (About 50% humid.)	35% H <sub>2</sub> SO <sub>4</sub> (About 65% humid.)	30% H <sub>2</sub> SO <sub>4</sub> (About 75% humid.)	18% H <sub>2</sub> SO <sub>4</sub> (About 90% humid.)	Control
1	1	0	0	0	0	0	0	0		0	0
	2	0.5	0	2.5	0	3.5	5	2		0	1
	3	40.5	44	52	47	61.5	52	64.5		34.5	52.5
	4	62.5	66.5	71.5	72.5	70.5	69	75		52	75.5
2 to 8 (ice chest)	1	0	0		0		0				0
	2	0	0		1		1				3
	3	36	27		59		47		3.5		45.5
	4	58.5	62		77		67		72		72
15	1	0	0		0		0				0
	2	0	0								0
	3	1	3		34.5		50.5		0		0.5
	4	14	13		67		75		34		40
Room temperature	1										
	2	0	0	0		12	0	0			0
	3	0	3.5	37		45.5	45	1.5	0		30.5
	4	12.5	20	67.5		75.5	66.5	30	1.5		71

lowered. In our experiments the seeds could be kept without injury at both low and high relative humidities at  $1^{\circ}$  C. It is not known to what degree this ability of the seeds to tolerate low relative humidities at low temperatures is due to a slower rate of moisture exchange between the seed and the surrounding medium or whether the seeds retain their vitality in lower water content at the lower temperature. In this connection it must be remembered that the vapor pressure of water is much lower at the low temperatures.

#### SECONDARY DORMANCY

Seeds (purchased from a seedsman) which had been stratified at  $1^{\circ}$  C. for 11 weeks<sup>2</sup> were left in the peat moss and transferred to temperatures of  $10^{\circ}$ ,  $15^{\circ}$ , and  $20^{\circ}$  C. These temperatures are favorable for germination but unfavorable for after-ripening. After four and eight weeks at these higher temperatures those seeds which had neither germinated nor become molded were returned to  $1^{\circ}$  C. The controls were kept at  $1^{\circ}$  C. continuously throughout the experiment. The germination data showed that the controls were much ahead of the seeds which had been for some time at the higher temperatures. Although the seeds had been almost ready to germinate when removed from  $1^{\circ}$  C., those which did not germinate at the higher temperatures when returned to  $1^{\circ}$  C. required an after-ripening period about as long as if they had never been stratified before. In other words, the seeds which were practically after-ripened when transferred to the higher temperatures were altered in such a way that a second after-ripening period at  $1^{\circ}$  C. was necessary.

In other experiments seeds were stratified 2, 4, 6, 8, and 10 weeks at  $1^{\circ}$  C., kept in peat moss and transferred for periods of 1, 2, 4, and 9 weeks to  $20^{\circ}$  C. and then returned to  $1^{\circ}$  C. Seeds were also stratified 3 and 9 weeks at  $1^{\circ}$  C., taken from the peat moss, and stored dry at  $1^{\circ}$  C. and at room temperature for periods of 2, 6, and 12 weeks and then re-stratified at  $1^{\circ}$  C. In every case the seeds required a longer stratification period the second time than the controls kept continuously at  $1^{\circ}$  C. Often there were indications of injury to the seeds while kept at the higher temperatures as shown by the fact that some of them became molded under these conditions and by the fact that the total germination reached after subsequent stratification at  $1^{\circ}$  C. was never as high as that of the controls.

Partially or entirely after-ripened seeds if taken out of the peat moss and stored dry at high or low temperatures or partially after-ripened seeds kept moist at higher temperatures require for germination a second low temperature stratification which is usually longer than the original stratification period. Such a need of a second after-ripening period has been de-

<sup>2</sup> As stated earlier in the paper seeds received from seedsmen are slower to germinate than seeds collected at Yonkers.

scribed by Crocker (2) as due to the development of secondary dormancy resulting from a change in the seed by which it becomes less responsive to germinative conditions. No evidence has been obtained as to the nature of the changes taking place when these partially or entirely after-ripened seeds are kept at the higher temperatures.

When seeds were stratified for periods of from 4 to 20 weeks at 10°, 15°, and 20° C. and then put into 1° C., the after-ripening period required was neither shortened nor lengthened.

## FACTORS IN DORMANCY

### WATER RELATIONS

In the dormancy of these seeds the seed coat as well as the embryo must be considered, for even when the embryos are after-ripened, the germination of the seeds is delayed or prevented by the seed coats. The nature of the germination of excised embryos when germinated without previous after-ripening as well as the degree of after-ripening at various periods of stratification at all temperatures can easily be studied.

### *After-ripening of Embryos*

The excised embryos of seeds which had been stratified two, three, and four and one-half months at temperatures of -5°, 1°, 5°, 10°, 15°, and 20° C., and -5° to +5° C. alternated weekly, were placed at room temperature on moist filter paper in petri dishes and in tap water through which a current of air was passing. Twelve embryos were used in each test. Only those embryos from seeds stratified at 1° and 5° C. produced seedlings when placed for two days on moist paper while those from the other temperatures and from the controls remained unchanged. Such embryos from low temperature stratifications for two months are illustrated in Figure 2. For a two months' temperature stratification period, 1° C. is best as far as the after-ripening of the embryo is concerned. Seedlings on moist paper were not produced by the embryos from the higher temperature even from those seeds which had been stratified four and one-half months. Harrington (12, p. 158) states that apple seeds which had been incubated for a considerable time under germination conditions unfavorable for after-ripening and which were still incapable of germination in intact condition, germinated when the seed coats were removed. The above experiments showed that this is not the case for *Sorbus* seeds as far as the temperatures tested are concerned.

To study further the after-ripening of the embryos at 1° C., seeds were stratified for periods of two, four, six, and eight weeks and the excised embryos studied as in the previous experiment. For each test 12 embryos were used. After the embryos had been kept on moist paper for three days, the control and the seeds stratified for two weeks showed no change. Those



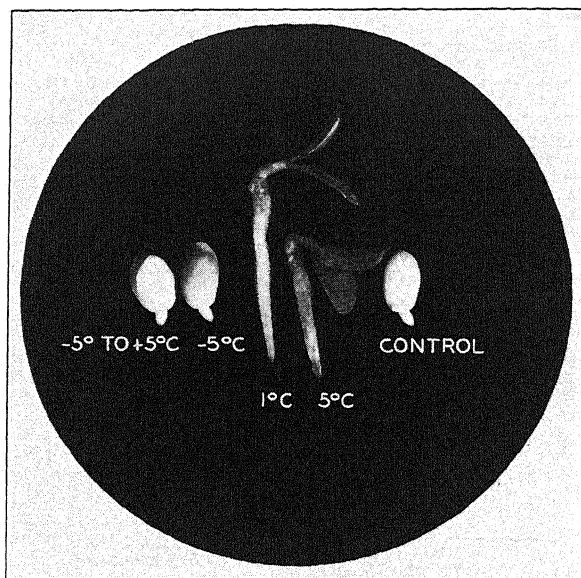


FIGURE 2. The degree of after-ripening attained by seeds which had been stratified for two months at various temperatures, as shown by the changes in the excised embryos after being on moist filter paper for two days.

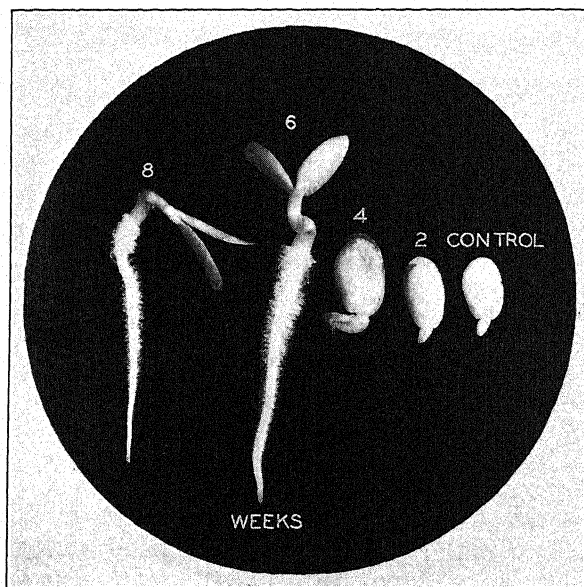


FIGURE 3. Excised embryos of seeds which had been stratified for various weeks at 1° C. after three days on moist filter paper.

stratified for four weeks showed a lengthening of the hypocotyl and a development of root hairs, while those stratified six<sup>3</sup> weeks and eight weeks produced fine seedlings. Typical examples from the different lots are illustrated in Figure 3. Twenty-one per cent of the seeds stratified eight weeks had germinated before the embryos were excised. The first to germinate are probably more vigorous and this explains why seedlings from seeds stratified six weeks appear to be in a better condition.

*Behavior of Dormant Embryos*

In both these experiments and the experiments with stratification at other temperatures described above, embryos which did not germinate were kept on moist paper for about three weeks. In many cases those coty-



FIGURE 4. Excised embryos of control seeds showing the development of the cotyledon in contact with the moist filter paper for 21 days.

ledons next to the moist paper enlarged to several times their original size, while the other cotyledons of the same seeds not in direct contact with the wet medium were unchanged. The enlarged cotyledons were green if exposed to light and yellow if kept in the dark. In Figure 4, two controls are

<sup>3</sup> The period of after-ripening of embryos is somewhat longer in the case of seeds obtained from seedsmen.

shown which had been kept for 21 days on moist paper. Non-after-ripened seeds do not produce seedlings when placed on moist paper; but when put in aerating water 20 to 40 per cent of seedlings are produced in 14 days.

It is important to note that the controls on moist paper never produce seedlings, that is, growth of both the hypocotyl and the cotyledons. They show some germination when their water and oxygen supply are furnished by aerating water but growth is very much slower than in the case of after-ripened embryos. The fact that it takes the cotyledons in contact with the wet medium several weeks to enlarge, the fact that there seems to be no movement of water to the other cotyledon not in contact with the wet medium, and the fact that some seedlings are produced in aerating water where the embryos are exposed to water on all sides, show that in the dormant embryos there is a very slow rate of water absorption and poor water movement. This slow water absorption and poor water movement is probably of fundamental importance in this type of dormancy. No doubt other factors are involved.

About 75 per cent of after-ripened excised embryos when placed on moist paper in petri dishes at room temperature developed into sturdy, vigorous seedlings, attaining a growth of 5 to 12 mm. in two days, and 25 to 40 mm. in six days. The dormant excised embryos behaved quite differently when placed under the same conditions. They did not develop into seedlings, and only the cotyledons adjacent to the moist paper enlarged and developed chlorophyll. When after-ripened embryos were placed in aerating water at room temperature, about 65 per cent developed into seedlings which elongated 12 to 25 mm. in two days and were about 40 mm. long in six days. About 20 per cent of the dormant embryos under the same conditions developed very slowly into seedlings which attained a maximum root growth of 10 to 15 mm. in about 14 days but which did not produce plants when placed in soil. Thus, slow water absorption and poor water movement are not the only factors involved in this type of dormancy for the seedlings developed from dormant embryos were not like the sturdy vigorous seedlings produced from after-ripened embryos, but were very slow in their growth and after a time reached a maximum growth of 10 to 15 mm. without ability to produce seedlings in soil. In Figure 5 are illustrated excised and after-ripened embryos after three days on moist filter paper and after three days in aerating water. It is seen that many of the after-ripened embryos have developed into small seedlings while no change can be noticed in the dormant embryos.

The use of excised embryos both when put in aerating water or placed on moist filter paper proved to be a good method for following the progress of the after-ripening of these seeds. Such methods should also be valuable in studying the after-ripening of other seeds.

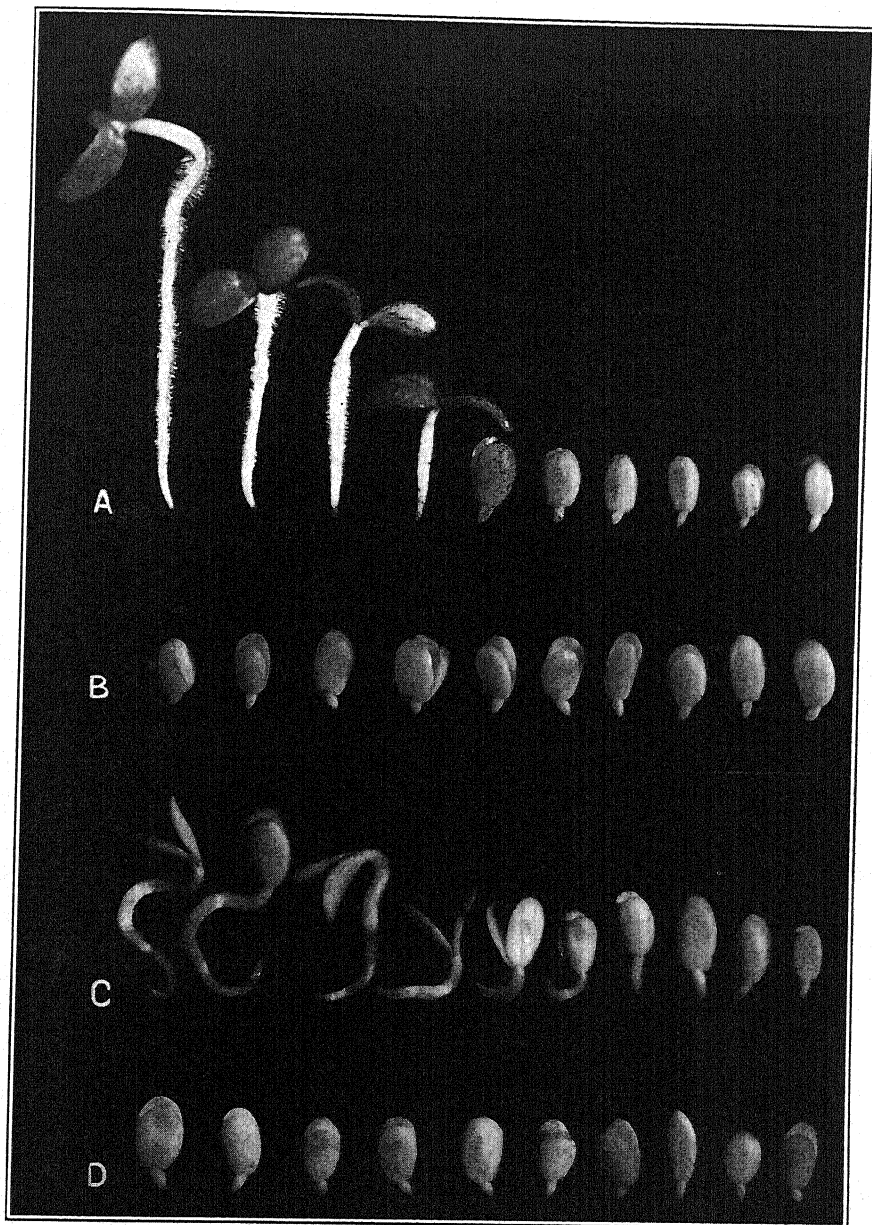


FIGURE 5. Behavior of excised after-ripened and dormant embryos after three days on moist paper: A, after-ripened embryos; B, dormant embryos. After three days in aerating water: C, after-ripened embryos; D, dormant embryos.

### *Rôle of Seed Coat*

That the seed coats also play a rôle in the dormancy is shown by the fact that the intact seeds do not germinate before two to four months of stratification at the optimum constant temperature of  $1^{\circ}\text{C}.$ , while the embryos after-ripen in six weeks' stratification under the same conditions. Further stratification after the embryo is after-ripened produces changes which result in the embryo's breaking through the seed coat and producing a seedling in the usual manner. This further stratification period may produce changes in the seed coat or give added vigor to the embryo so that it can break through the seed coat, or it may produce changes in both the embryo and the seed coat.

### ENZYME STUDIES

In order to obtain some information as to the processes going on in the seed during the period of low temperature stratification, the activities of various enzymes were determined at intervals throughout the after-ripening period.

#### *Catalase*

The material was prepared for enzyme determinations by taking a definite number of seeds which had been stratified the desired length of time and grinding them in a mortar with sand and an excess of calcium carbonate and making up to a suitable volume in a volumetric flask. For the estimation of catalase, aliquots of these solutions were taken and the catalase activity measured by the amount of oxygen liberated from hydrogen peroxide ( $\text{H}_2\text{O}_2$ ), using the apparatus and procedure described by Davis (5) except that the hydrogen peroxide was neutralized by calcium carbonate instead of sodium hydroxide. Preliminary tests showed that if the samples were taken in such a way as to include in suspension the ground-up seed material the results obtained for catalase activity were only slightly higher than those obtained when the material was first centrifuged and aliquots of the supernatant liquid were used. This difference amounted to only a few per cent and tests with seeds throughout the stratification period showed that this difference did not change while the seeds were after-ripening. It was, therefore, concluded that no additional information would result from determining both soluble and insoluble catalase.

*Various weeks at  $1^{\circ}\text{C}.$*  The upper curves in Figures 6 and 7 show the catalase activity of seeds stratified for various lengths of time at  $1^{\circ}\text{C}.$  The lots of seeds were so stratified that samples which had been stratified for a different number of weeks could be had the same day and determinations made under exactly comparable conditions. The values as plotted were obtained by taking the activity of unstratified seeds as 1.00 and calculating the activity of the stratified seeds in terms of these controls. The catalase

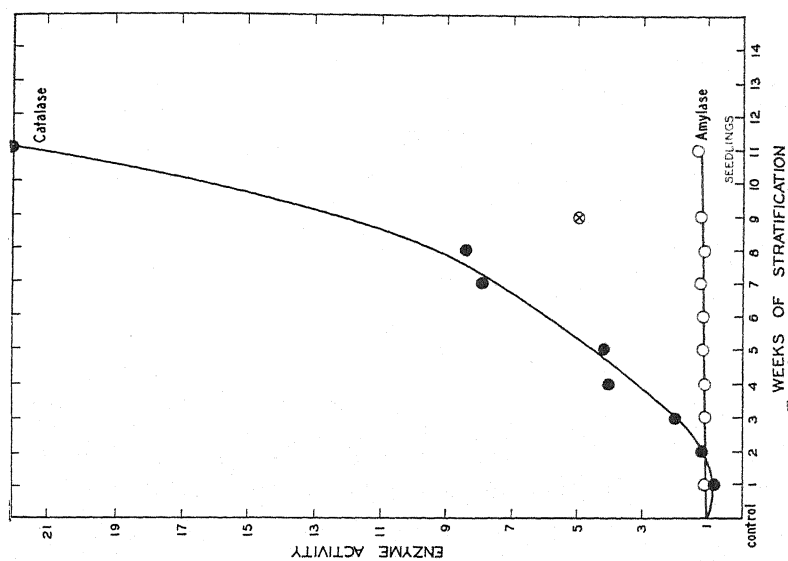


FIGURE 7. The catalase and amylase activities of seeds after various weeks of stratification at 1° C. and of seedlings produced in stratification.

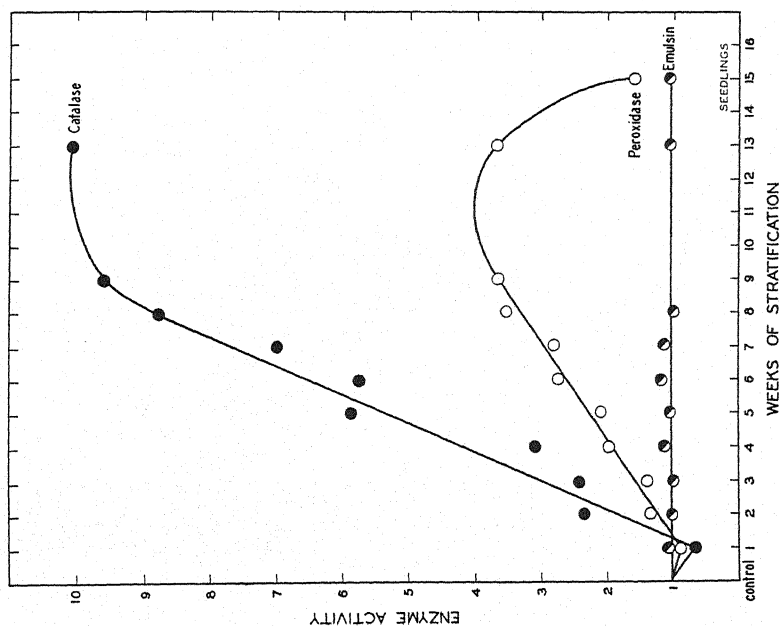


FIGURE 6. Changes in the catalase, peroxidase, and emulsin activities of seeds while after-ripening at 1° C.

curve in Figure 6 was obtained from the amounts of oxygen liberated by 15 seeds (a 5 cc. aliquot of a 100 cc. solution made up from 300 seeds) in five minutes, and the curve in Figure 7 from the oxygen liberated by 30 seeds (a 5 cc. aliquot of a 50 cc. solution made up from 300 seeds) in one-half a minute.

It is seen from the curves that the catalase increases from week to week during the after-ripening period. In the case of Figure 6 after two weeks of stratification the catalase was more than twice that of the unstratified seeds and after nine weeks it had attained an activity about nine and one-half times that of the control. A similar catalase increase is shown in Figure 7 where a value for the seedlings is included. The seedlings were obtained from seeds of these lots which had germinated to the extent of putting forth a hypocotyl of from one to four millimeters in length and were obtained from lots which had been stratified a considerable period (10 to 12 weeks). The seedlings represented in Figure 7 had an activity about 22 times that of the unstratified seeds. The value for nine weeks indicated in Figure 7 by a cross is not included in the curve because some of the seeds in this lot had already germinated at this time and the value of 5.00 was obtained from seeds which had not germinated. This value is, therefore, not directly comparable with the values for the seeds from the other weeks since those values represent the whole sample while that obtained for nine weeks represents only the less vigorous seeds as the more vigorous ones had already germinated. The data show that there is an increase in the catalase activity of the seeds from week to week when stratified at 1° C. which is the optimum constant temperature for germination. Increase in catalase activity during the after-ripening period of seeds has also been found by Eckerson (10) and Sherman (19) with *Crataegus*, by Davis (4) with *Cornus florida*, by Davis (6) with *Ambrosia trifida*, by Jones (15) with *Acer saccharum* Marsh., by Pack (17) with *Juniperus*, and by Rose (18) with *Tilia americana*.

*Other temperatures.* To learn more about any possible correlation between catalase activity and after-ripening, catalase changes were followed in seeds stratified at temperatures less favorable for germination and these values compared with the changes produced at 1° C. Seeds were stratified so that on a given day samples could be obtained which would represent various weeks of stratification at various temperatures. In Figure 8 are shown the catalase activities of such seeds, the catalase activities being plotted directly as cc. of oxygen liberated in one minute by aliquots representing 25 seeds. Activities of seeds stratified for 1, 3, 5, 7, 9, 11, 13, and 15 weeks at 1°, 5°, ice box temperature, 10°, 15°, and 20° C. are shown.

Stratification at 10°, 15°, and 20° C. resulted in little change in catalase activity. At 10° C., however, there was an initial rise during the first few weeks followed by a slight drop and then the catalase remained more or

less constant instead of increasing further as at  $1^{\circ}\text{C}$ . The largest catalase increase in this experiment occurred in the case of the seeds stratified in the ice box which also showed the best germination. The temperature of the ice chest was maintained by brine from an ammonia system and it represents a fluctuating temperature (about  $2^{\circ}$  to  $8^{\circ}\text{C}$ .). After 14 weeks of strat-

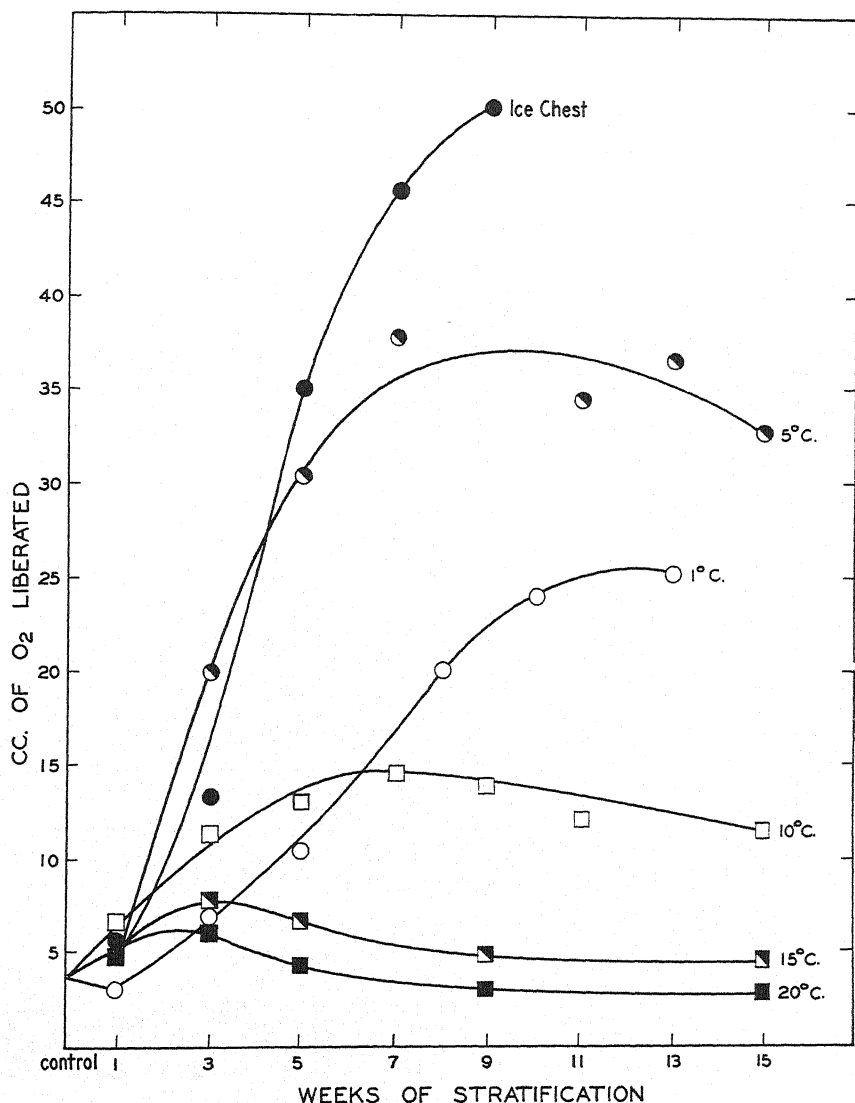


FIGURE 8. The catalase activities of seeds stratified for periods of 1 to 15 weeks at various temperatures.



ification 32.4 per cent had germinated in the ice box and 26.4 per cent at  $1^{\circ}$  C. At  $5^{\circ}$  C., 0.4 per cent had germinated in six weeks and no further germination was obtained. No germination had resulted in any of the other temperatures. It will be recalled that in most cases when seeds are stratified at  $5^{\circ}$  C. there is a small percentage of early germination, that is, a small number germinate before there is any germination at the favorable temperature of  $1^{\circ}$  C. The catalase values of seeds stratified at  $5^{\circ}$  C. are interesting in this connection since they show a marked initial increase in catalase activity and then more or less of a standstill or perhaps a decrease. It is possible that the first five or six weeks at  $5^{\circ}$  C. may be quite favorable for after-ripening and then something takes place which is deleterious, hence there is lack of any further catalase increase and lack of germination. This point needs further study.

*Alternating temperatures.* Seeds were also stratified at various alternating temperatures, as for example  $1^{\circ}$  to  $5^{\circ}$  C. weekly,  $1^{\circ}$  to  $5^{\circ}$  C. daily,  $1^{\circ}$  to  $10^{\circ}$  C. daily,  $1^{\circ}$  to  $10^{\circ}$  C. weekly,  $1^{\circ}$  to  $15^{\circ}$  C. weekly, etc., and catalase activity and germination recorded. In many of these conditions excellent germination resulted and there was also a marked increase in catalase activity. The correlation between catalase increase and germination, however, was not perfect as some conditions gave good catalase increase and poor germination. The catalase rise at  $5^{\circ}$  C., for example, was always greater than at  $1^{\circ}$  C. although very little germination resulted at this temperature. Under no condition did germination take place without a considerable increase in catalase activity.

When seeds are stratified under conditions favorable for after-ripening, and therefore for germination, there is an increase in catalase activity from week to week until germination occurs. The catalase activity of the seedlings produced is much higher than that of the fully after-ripened seeds. A stratification condition which causes an increase in catalase is, however, not necessarily a good condition for germination.

#### *Other Enzymes*

*Peroxidase.* The middle curve in Figure 6 shows the changes in peroxidase activity of the seeds as they after-ripen. Peroxidase was determined by measuring the purpurogallin formed from pyrogallol at a pH of 6.5 (8, p. 492). Aliquots of the same solutions as were prepared for catalase determinations were used. The peroxidase activity is quite low and it was necessary to use large aliquots and allow the reaction-mixture to stand for about a week in order to have enough purpurogallin formed for colorimetric comparisons. It is seen from the curves that the peroxidase changes, while not so great, parallel very closely the catalase changes up to the time of germination where catalase increases markedly and peroxidase falls.

*Emulsin.* When the seeds are ground the odor of benzaldehyde resulting

from the hydrolysis of amygdalin at once becomes apparent. Tests showed that the emulsin content is very high. Results from quantitative determinations of emulsin activity as measured by rate of hydrolysis of amygdalin are shown in the lower curve of Figure 6. The rate of hydrolysis was determined by measuring the reducing sugars resulting from the action of emulsin on amygdalin. No change in emulsin activity was found as after-ripening progressed and germination began.

*Amylase.* The lower curve of Figure 7 shows the amylase activity of seeds from various weeks of stratification at 1° C. as measured by the reducing sugars formed with soluble starch as a substrate. Results of this test indicate no appreciable changes in the activity of this enzyme during the after-ripening period or even during the early germination period.

These preliminary tests indicate that emulsin and amylase do not change in activity during the after-ripening period while peroxidase increases in a manner analagous to catalase.

#### SUMMARY

1. The seeds of *Sorbus aucuparia* L. are dormant and require an after-ripening period for germination to take place. This after-ripening takes place at 1° C. in from two to four months and in about the same time at alternating temperatures of from 1° C. to 5° C., alternated daily or weekly. When planted out-of-doors in cold frames in the fall best results are obtained when placed in frames with a board cover, the seedlings resulting the following spring.<sup>4</sup> In all cases, for best results the seeds should be removed from the berries as soon as possible after they are ripe.

2. Untreated granulated peat moss, pH of about 4, was found to be superior as a stratification medium to sand, muck, or peat moss adjusted to more alkaline or more acid pH values.

3. Attempts to break the dormancy of these seeds by treatments with various chemicals were unsuccessful.<sup>4</sup>

4. Subjecting the seeds to high pressures or to ultraviolet rays and electro-magnetic waves did not induce germination.

5. When seeds were kept under ordinary dry storage conditions at room temperature for six months they partially after-ripened so that the after-ripening and germination period required at 1° C. was somewhat shortened. No other storage condition was found under which the seeds after-ripened to a greater extent.

6. Seeds stored for two years in sealed or unsealed containers, and

<sup>4</sup> Since this article was written a paper by L. Fabricius (Forstwiss. Centralbl. 53: 413-418. 1931) has appeared in which out-of-doors plantings are also recommended for seedling production. In the spring following his fall plantings, he obtained about 35 per cent germination. Treatments with chemicals such as hydrochloric, sulphuric, and nitric acids, sodium chloride, pepsin, and pancreatin were unsuccessful in inducing germination.

under vacuum, at temperatures ranging from  $-8^{\circ}$  C. to room temperature, were just as viable as freshly harvested seeds. When stored at  $25^{\circ}$  C., either sealed or unsealed, the vitality was rapidly lost.

7. Seeds stored at room temperature over concentrated sulphuric acid or anhydrous calcium chloride were injured as well as when stored at 50 per cent and 75 per cent humidity. Seeds stored at 25 per cent humidity were not affected. At lower temperatures the seeds tolerated both higher and lower relative humidities than at room temperature.

8. When partially or entirely after-ripened seeds are stored dry at high or low temperatures or when partially after-ripened seeds are kept moist at higher temperatures, a secondary dormancy is produced so that the seeds require a second low temperature after-ripening period which is usually longer than the original stratification period.

9. Tests with excised embryos showed that the embryos are after-ripened after six weeks of low temperature stratification even though germination of intact seeds does not take place until considerably later, thus showing that the seed coat plays a rôle in the dormancy of these seeds.

10. Dormant embryos when placed on moist filter paper absorb water very slowly, the cotyledons in contact with the paper enlarge only after several weeks. Water movement in the embryo is extremely slow so that the upper cotyledon, not in contact with the moist medium, does not enlarge. Under the same conditions after-ripened embryos produce seedlings in a day or two. When placed in aerating water there is some development of dormant embryos; however, such embryos will not produce plants.

11. The catalase activity of seeds stratified under various conditions favorable for after-ripening increases from week to week until the seeds are fully after-ripened when it is about ten times that of the dry controls. The catalase activity of the seedlings produced is about twenty times that of the controls. A stratification condition which causes an increase in catalase is, however, not necessarily a good condition for after-ripening, as is shown at  $5^{\circ}$  C. No condition was found, however, under which germination took place without an increase in catalase activity. At the higher temperatures of  $10^{\circ}$ ,  $15^{\circ}$ , and  $20^{\circ}$  C., which are unfavorable for after-ripening, there was an initial increase in catalase activity during the first few weeks which was then followed by a drop.

12. Preliminary tests indicate that peroxidase activity increases in a manner analogous to catalase except in the case of the seedlings in which it is lower than that of the after-ripened seeds. Emulsin and amylase do not seem to change in activity with after-ripening and germination.

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## COTTON FIBERS. I. ORIGIN AND EARLY STAGES OF ELONGATION

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Mature cotton fibers present a high degree of both intra- and inter-varietal differentiation. The variance of opinion concerning their origin and subsequent development provides no morphological basis for their natural grouping. As an aid to future classification of mature fibers, a study covering the entire period of fiber growth has become necessary. In this first paper of a series only the origin and early stages of fiber elongation are discussed.

The process of fiber formation from the surface of the cotton seed has had a limited but somewhat controversial treatment by various authors during the past fifty years. Bowman, in a series of lectures published in 1881, presented the first detailed account. In describing the process of fiber elongation he said (6, p. 25), "the method of growth is by successive linear development of cells, the cell wall at the point of junction being gradually absorbed until an exceedingly elongated cell is produced which constitutes the cotton fiber." With the exception of one point, the ideas of the same author were essentially unchanged in a book published almost thirty years later. Intervening studies had shown the fiber to be a unicellular instead of a multicellular structure. The later description of the seed coat during early stages of fiber development is given (7, p. 87) as follows:

"As soon as fertilisation takes place structural differences in these several layers begin to be apparent . . . and also the active parenchymatous cells which lie below the cuticular layer, commence to elongate and to force themselves up through the cuticle, when they appear above the surface as elevations or buds which form the base of the fibers now first generated."

" . . . Some of the cells in the fibril layer commence to extend, and from a compressed circular form become oval, with the longitudinal axis at right angles to the surface of curvature of the seed." (7, p. 88) . . . "As the young fiber by its growth is pushed out they are compressed together by filling the interior of the seed pod with a tangled plexus of unicellular hairs in various stages of growth."

" . . . The first appearance of the young fiber buds always occurs on the

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surface of the fibril layer at the end of the ovule farthest removed from the point of attachment of the ovule to the placental matrix, and from here spread over the whole surface, and are always the longest at this end, gradually becoming shorter as the point of attachment is reached so that at the root end of the seed there is only a scanty growth. . . . This is the birth of the cotton fiber."

The description as quoted is somewhat ambiguous as to the exact layer of cells in the outer seed coat from which the fibers arise. Later, in explanation of a text figure (p. 89, Fig. 27) we find more definitely, "Young cotton fibers having their origin in the second layer of cells beneath the cuticle, forcing themselves up through it."

Although no definite statements are made concerning the period of time during which the initiation of fiber growth takes place, we might infer, from the reference (p. 88) to a later stage in which the interior of the seed pod is filled with hairs in various stages of growth, that he believed the process to continue over a number of days after fertilization.

Monie (15, 16) described the process of fiber formation as follows (15, p. 23). "The construction is effected by the successive formation and deposit of a number of cells, one above another, on the dermal covering of the seed inside the cells of the capsules. As growth progresses the parts of the walls of these cells which are in contact with one another begin to corrode, and this corrosion goes on until they gradually become absorbed and disappear entirely, leaving one long extended cell or fiber behind."

Flatters (11, p. 59-65) states that the fiber is a cuticular outgrowth of the ovule and that all of the fibers are not developed at the same time on the same ovule, the latter fact accounting in part for the great variation in fibers from the same seed.

Balls (1) contemporaneously with Flatters, and also in subsequent publications (2, 3, 4), has presented the most extensive discussion of fiber development. Concerning the early stages he says (4, p. 17), "The hair is formed by the outward extension of a single cell from the epidermis of the seed coat. . . . The sprouting of such individual cells of the epidermis is not identical although they all sprout at the same stage of boll development, on or near the day when the flower opens. Some cells grow to full-sized lint hairs, others to shorter lint, others elongate as little as thirty-fold, and so form the fuzz hairs."

In another publication (2, p. 82-83) the time of initiation of fiber growth is further restricted by the statement, "The density of lint on the boll is determined when the lint first originates by the protrusion of individual epidermal cells. There does not appear to be any further growth of epidermal cells into lint hairs after the first day in spite of accepted statements to the contrary. The density of the lint on a given area of seed coat

should, thus, other things being equal, depend on the circumstances of the environment on the day when the flower opens."

A still later publication (3, p. 7) presents drawings of the first few days of fiber development which show clearly that the fibers originate in the epidermal layer, and also indicate, in support of his earlier contention, that growth of new fibers is not initiated in this layer after the first day.

The idea that the initial differentiation of fiber cells from epidermal cells takes place only upon the day of flowering remained uncontested until 1929 when Turner (19) presented a critical analysis of Balls' conception. It was pointed out that if the latter's findings were correct, the total number of fibers per seed would remain constant, and that the number of fibers per unit surface area would continuously decrease as the volume and surface of the seed increased. Gulati, working in Turner's laboratory, had found that there was an approximate increase in numbers of fibers upon the seed from 4530 at the time of flowering, to 16,420 twenty-eight days later, and an increase of non-fiber-forming cells from 22,990 to 50,610 during the same length of time. Turner suggested that if further differentiation of epidermal cells to form fibers after the first day were conceded, it would account for the fact that fibers of various lengths are found even within a small area of the surface of a single seed. From Gulati's results it seemed probable that both epidermal and hair cells increase largely up to three or four weeks after the flowering date.

In reviewing Turner's discussion, Harland (13) found difficulty in reconciling the ideas with anatomical observations made by Barritt (5). The last author had reported no development of the bases of hair cells after differentiation, enormous development of neighboring epidermal cells during the same period, and consequent lateral pressure upon the hair-cell bases. This force, together with wall thickening, was said to close the lumen at an early age. Harland suggested that if fiber growth initiation extended over a considerable period of time, the younger hairs should be recognized by differences in the degree of this constriction. In the absence of anatomical evidence that hairs in all stages of development exist, he preferred to allow the balance of evidence to remain with Balls.

In reply to Harland's criticism Gulati (12) presented values for the number of fibers upon corresponding areas of seed coats from the first to the fifth day from flowering and found an increase from 2208 to 14,809. He also reported that epidermal cells from ovules of *Gossypium sanguineum* and *Gossypium cernuum* had been seen in the process of division in the first, fourth, seventh, and tenth day ovules; that hair cells of greatly different lengths had been observed in close proximity to one another, that some cells appeared to have arisen sometime previously; that in addition to single outgrowths on the seventh and tenth day ovules, there were many cases of two, three, and four adjacent hair cells sprouting simultaneously



in a group; and that the constrictions at the bases of the hair cells were no more pronounced on the twentieth day than on the tenth and therefore could not be used as a basis for determining the age of the hairs.

Hawkins and Serviss (14) reported the origin of fibers of Pima (*Gossypium barbadense*) and Acala (*Gossypium hirsutum*) in the epidermal layer of the ovule at the time of fertilization. Their measurements of fiber development after the third day were made macroscopically, *en masse*, and no mention is made of continued fiber growth initiation in epidermal cells.

Support of Turner's hypothesis and of the correctness of Gulati's observations has come through the study of *Gossypium hirsutum*, *Gossypium indicum*, and *Gossypium neglectum* during the first 48 hours of development. Singh (18) found that fibers on the first day are sparsely distributed over the outer integument, that upon seeds 36 hours after fertilization cotton fibers in different stages of development occurred, and that upon seeds 48 hours after fertilization almost all epidermal cells are involved in the formation of fibers.

The process of cotton fiber formation is essentially a cellular phenomenon covering two of the three phases of growth, in its technical sense, in a most striking fashion. *Cell enlargement* continues until the mature fiber often attains a length two thousand times as great as its diameter. *Cell differentiation* in this instance consists in the deposition of quantities of cell-wall material, largely cellulose, until the cell lumen is reduced to a small fraction of its original diameter. We may assume that the well developed fibers of any particular variety represent the most complete expression of these two phases of growth activity. It may then follow that the large number of fibers from the same seed, varying in size, form, and possibly consistency, from the "normal" are the result of combinations of these processes in varying degrees.

#### MATERIAL AND METHODS

The material chosen was *Gossypium hirsutum* L. Strain 4 of a representative, pure bred, American cotton, whose reputation for wilt resistance had been established. The plants were grown at the South Carolina Experiment Station, Clemson College, South Carolina, during the summers of 1929 and 1930. For several years previous to this time the nutritional needs of this same variety had been studied upon the same ground by members of the experiment station staff. It was possible, with the resulting information at hand, to treat the soil in such a way as to maintain unusual uniformity in growth. Adequate protection from injurious insects and plant diseases resulted in a high percentage of vigorous plants. It is probable that all of these precautions, along with the artificial self-pollination of all of the flowers, contributed to the control of variation frequently caused by environmental differences, and made possible the col-

lection of unbroken series of stages from the standpoints of boll, seed, and fiber development.

During the early period of profuse flowering from 300 to 500 flowers were self-pollinated and marked daily with dated tags. Later the number was reduced to 100 to 150 flowers daily. In consideration of the large

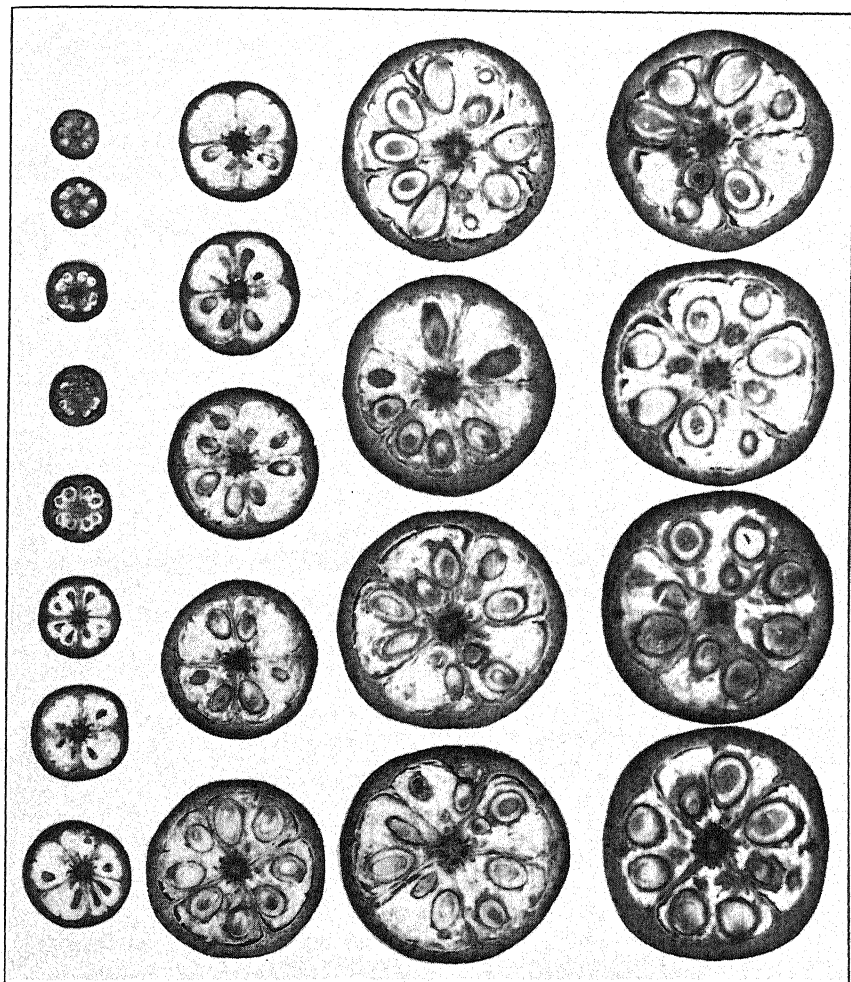


FIGURE 1. Median cross-sections of bolls of *Gossypium hirsutum* L. showing daily increase in size from the date of flowering to the twenty-first day of development.  $\times 1 \frac{1}{8}$ .

number of young bolls which are usually shed, the supply of material was no more than adequate for the needs of the experiments. At the end of a period of 45 to 50 days it was possible to collect every daily stage of bolls of

known age from flowering to maturity at any given time. For the studies of fiber development, 20 bolls were collected for each daily age and from these a representative specimen was taken. Bolls thus selected, when arranged in a series, presented gradations in size indicated by their cross-sections at median points (Fig. 1).

For the first five or six days following flowering it was possible to study the fibers in fresh material by means of free-hand cross-sections of the small seeds, made with a safety-razor blade. Sections from 15 to 25 microns in thickness showed clearly the early stages of elongation of epidermal cells of the seed coat (Figs. 2 and 3). The mounting medium in which these

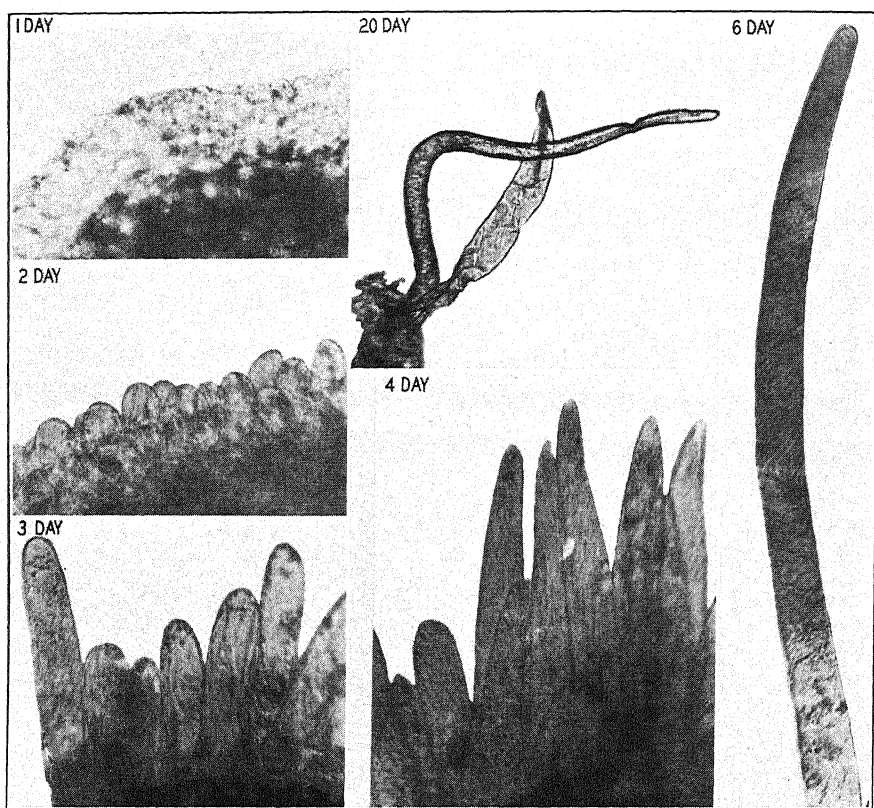


FIGURE 2. Photomicrographs of living fibers of *Gossypium hirsutum* upon the date of flowering and at intervals during the first twenty days of boll development.  $\times 355$ . particular photographs were made was a mixture of equal parts of tap water and filtered cell sap. Large quantities of sap may be easily obtained by subjecting a mass of fibers to pressure. The cells remained in an excellent state of preservation in such mounts, cytoplasmic movements fre-

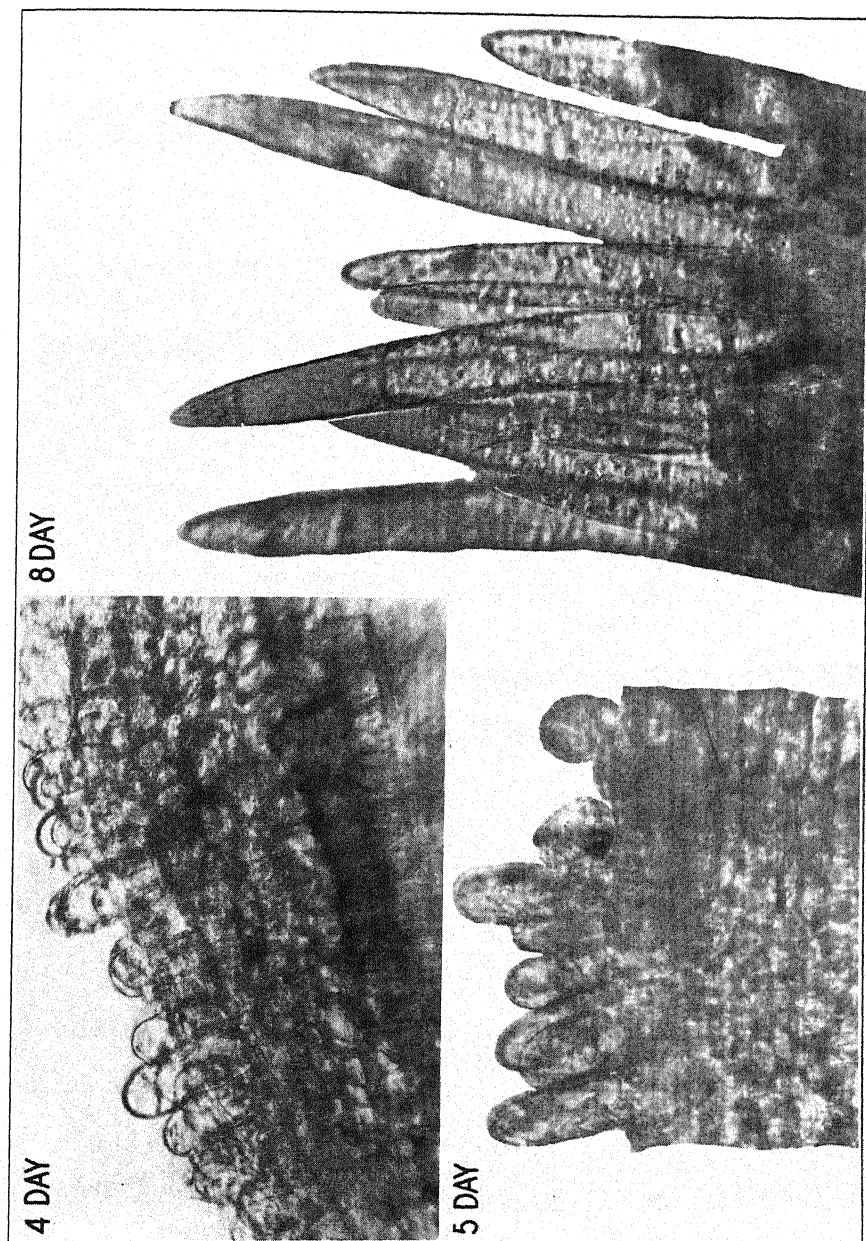


FIGURE 3. Photomicrographs of living fibers of *Gossypium barbadense* L. at four, five, and eight day intervals following the date of flowering.  $\times 625$ .

quently continuing for from three to four hours after the preparation was made. Drying was prevented by sealing the cover glass with a narrow band of paraffin, a small pore being left at one side for aeration. If a mount so prepared were placed upon the stage of a microscope turned to horizontal position, with the pore on the upper edge of the cover glass, the air bubbles which formed abundantly in the mounts of living cells often rose toward the pore and escaped, thus improving the conditions for both observational and photographic purposes. The cloudiness of some of the earlier mounts (Fig. 1) was found to be due to coloration of the cell sap of the cut cells and also the expressed sap in which the material was mounted. It was partially eliminated by the successful use of pure tap water as a mounting medium (Fig. 2).

Preservation of seeds with fibers attached at any stage of development in a solution of 1.4 per cent formalin and 70 per cent alcohol produced no appreciable distortion. It was found that fibers so treated could be successfully mounted in paraffin oil for microscopic observation. Living fibers were later mounted in paraffin oil with excellent results. The entire lock of fresh material was dropped into enough paraffin oil to completely cover it. The seed selected was then carefully separated from the adjacent seeds with fine dissecting needles. Sectioning and mounting in a drop of paraffin oil then followed. Denham (10) has used paraffin oil as a mounting medium for mature cotton fibers. Schaede (17) used paraffin oil for mounts of living cells of *Allium cepa*, *Hyacinthus romanus*, and *Tradescantia virginica*. He found that cell-division of the pollen mother cells continued without interruption in the latter form when so prepared. Dawson and Belkin (9) discussed the tendency of protoplasmic surfaces to be wetted by liquid paraffin, olive oil, etc. Chambers and Höfler (8) have more recently observed that liquid paraffin adheres so strongly to a tonoplast of *Allium cepa* that it cannot be removed without rupturing the membrane or carrying a portion of the tonoplast away with it. Because of the prevention of drying in the mounts, the absence of distortion in the cells and the clearness of vision obtained, paraffin oil proved to be the most satisfactory mounting medium used in the present study.

For stages later than the fifth or sixth day from flowering, cross-sections of the seeds were no longer possible because of injury to the very delicate, much elongated fibers. Mounts were then obtained by lifting a diminutive portion of the seed-coat with the point of a sharp knife made by securing a small wedge-shaped section of a safety-razor blade in a wooden handle. Cross-sections as well as pieces of seed-coats with fibers attached were taken from median regions of the longitudinal axis of the seeds. The particular seeds used in all stages were always one of the two in the second position from the tip of the boll in a well developed lock. The present account is confined to observations in connection with epidermal cells of the

seed coat, fiber origin, and early stages of fiber elongation during the first seventeen days from the date of flowering.

## RESULTS

### GROWTH OF FIBERS FROM THE FIRST TO THE TWENTIETH DAY

During the earliest observations of living material many epidermal cells in the process of elongation were found upon the first day (Fig. 2, 1 day). On the following days (Fig. 2, 2 day, 3 day, 4 day, 20 day), in addition to the longest cells, there were many others whose length and cytoplasmic condition indicated that they had arisen later at successive intervals. In the longer cells the presence of vacuoles, large numbers of spherical bodies of high refractive index, and aggregates of granular as well as flocculent amorphous material contributed to the appearance of the coarse, uneven, cytoplasmic structure of a mature cell. The shorter fibers with dense, finely granular, homogeneous cytoplasm had the characteristics of a younger cell, and were similar in appearance to the early stages of growth of the longer fibers. The shape of the fiber tips in these early stages appeared to be a function of fiber length. The shorter fibers have broad, rounded tips, which became sharper as elongation progressed (Fig. 2, 3 day, 4 day, 6 day). On the sixth day (Fig. 2, 6 day), the fiber length had increased so that it was no longer possible to photograph it from base to tip at the same magnification, upon one 5×7 film. The picture shown was made upon two separate films and the prints from these were joined.

After the sixth day there was even more marked contrast in fibers of different lengths. The longer ones, through increased vacuolation, became more and more hyaline, and the shorter ones, in addition to their usual cytoplasmic density, were rendered more and more opaque by the apparent presence of a pigment in the cell sap. An attempt to illustrate this contrast is made in Figure 2, 20 day. The turgid, entire fiber was only slightly injured near the tip by approximately the same amount of manipulation that had caused the complete collapse of the greater portion of the larger fiber. This differential resistance to fiber injury may be a membrane effect, possibly augmented by the density of the cytoplasm in the shorter fiber, and not evidenced in the longer fibers of more aqueous cytoplasmic consistency.

For comparison, three stages of fiber development in *Gossypium barbadense* L. are shown (Fig. 3, 4 day, 5 day, 8 day). It may be observed that the rate of growth of these fibers as compared with those of *Gossypium hirsutum* (Fig. 2) is somewhat slower. The clearness of the mounts which were made in tap water also made distinguishable the individual cells in the parenchyma tissue immediately below the epidermal layer. In both Figures 2 and 3 a high percentage of epidermal cells are seen to be producing fibers. These observations, as well as those in succeeding stages of seed

and fiber development, made the probability of simultaneous growth initiation in the fibers more remote. The factors involved could not be satisfactorily accounted for upon the basis of differential growth rate.

#### SIZES OF EPIDERMAL CELLS IN RELATION TO SURFACE AREAS OF SEEDS

The definite size relationship which necessarily exists between the constantly enlarging cotton seed and its constituent tissues is probably expressed in its most simple form in the ratio between the increase in surface area of the seed and increase in size of the single layer of cells which make up its epidermal covering. The important cell axes in this connection are those which are tangential to the surface of the seed. The increase in surface area of the seed from day to day, as illustrated in Figure 1, cross-sections 1 to 21, must be balanced either by increase in magnitude of these parallel axes or by increase in the number of cells.

The relative increase in surface area of the cotton seed at any stage of development, as compared with its area upon the date of flowering, may be expressed mathematically in terms of the ratio of the squares of the averages of the major and minor seed axes. Because of a flattening which commonly occurs upon one side of the cotton seed parallel to the major or longitudinal axis, it is necessary to use values for two minor axes. Both minor axes may be taken perpendicular to the major axis at its middle point, the first minor axis being parallel to the flattened side, and the second minor axis perpendicular to it. Values so obtained are listed in Table I. Ratios of the squares of the averages of these values show a thirty-two-fold increase in surface area during the first twenty days. Growth continues to a point indicated for the mature (preserved) seed. It will be observed that the value for the mature seed (dried) is reduced to a ratio but slightly larger than that for the twentieth day (preserved). The values at maturity are given here mainly for the purpose of general comparison. Details of the stages of fiber development from the twentieth day to maturity will be published subsequently.

In order to determine the rate of increase in length of epidermal cell axes tangential to the surface of the seed, it seemed at first desirable to take the average of a number of such axes for each cell measured. Later it was found to be adequate, for the purpose of general comparison, to measure one axis each of twenty cells as they appear in a continuous row in the cross-sections of the seed-coat. Rates of increase thus obtained may be compared directly with ratios for increase in surface area of the seed. Values for increase in surface area of the seed shown in Table I indicate a ratio between the one day old and the twenty day old seed of 31.7. It would be necessary for the epidermal cells to increase transversely in the same proportion, if the balance in surface area of the seed and size of the epidermal coating were maintained through cell enlargement. In Table II,

the ratio of average increase between the transverse cell axes on the first day and on the twentieth day is shown to be 2.19. Increase in length of transverse axes is, therefore, not in proportion to the increase in surface area of the seed and will not account for the presence of a continuous coating of fiber-forming and non-fiber-forming cells upon the surface of the en-

TABLE I  
SIZES OF SEEDS\*

Age in days	Major axis	First minor axis	Second minor axis	Squares of the average of the three axes
1 (Preserved)	1.60	0.96	0.94	1.37
16 "	8.32	4.01	3.83	29.05
20 "	10.35	5.25	4.0	42.64
Mature "	11.54	6.70	5.66	63.52
Mature (Dried)	9.59	5.50	4.70	43.56

\* Averages for measurements of 30 seeds at each age.

$$\text{Ratios } \frac{\text{Squares of averages of 1st day}}{\text{Squares of averages of 20th day}} = \frac{1}{31.1};$$

$$\frac{\text{Squares of averages of 1st day}}{\text{Squares of averages of mature dried seed}} = \frac{1}{31.7}$$

TABLE II  
TRANSVERSE AXES OF EPIDERMAL CELLS\*

Age in days	Axes in microns	Age in days	Axes in microns
1	8.90	9	10.81
2	8.60	10	12.50
3	8.98	13	15.68
4	10.17	17	18.23
5	9.75	20	19.50
6	9.54	Mature (preserved)	36.67
7	10.17		
8	11.02	Mature (dried)	19.92

\* Averages of measurements of 20 cells at each age.

$$\text{Ratios } \frac{\text{Average axes of 1st day}}{\text{Average axes of 20th day}} = \frac{1}{2.19};$$

$$\frac{\text{Average of axes of 1st day}}{\text{Average of axes of mature (dried)}} = \frac{1}{2.23}$$

larging seed. The values for transverse axes of mature cells are again shown merely for the purpose of general comparison. The intervening stages of epidermal cell behavior will be published subsequently. The ratio of values for cell axes at maturity (dried) is but slightly higher than that for the twenty day old seed. These ratios compared with those in Table I would indicate a consistent relationship between seed size and size of epidermal cells throughout the entire period of growth.

If we assume the shape of the cotton seed to be that of a prolate spher-



oid, an idea of the approximate surface areas at different stages of development may be obtained in terms of square millimeters by means of the following formula:

$$S = 2\pi b^2 + 2\pi \frac{ab}{e} \sin^{-1} e$$

in which  $a$  and  $b$  are the major and minor semi-axes of the seed respectively and  $e$  the eccentricity, the value of which may be obtained in turn by the formula,

$$e = \sqrt{1 - \frac{b^2}{a^2}}.$$

Using for the major seed axes the values given in Table I on the first day and at maturity, and for the minor axes, at the same periods, the averages of the first and second minor axes respectively from the same table, the values obtained for  $e$  show the close similarity in proportion of the seed on the first day and at maturity. In terms of radians the value of the angle whose sine is  $e$  for the one day seed is 0.935 and for the mature seed is 1.005.

Applying the entire formula for surface area,

$$S = 2\pi(0.475)^2 + 2\pi \frac{(0.80)(0.475)}{0.805}(0.935) = 4.19$$

we find the approximate surface area of the 1 day seed to be 4.2 square millimeters or 4,200,000 square microns.

Similarly for the mature seed,

$$S = 2\pi(2.55)^2 + 2\pi \frac{(4.795)(2.55)}{0.844}(1.005) = 132.34$$

the surface area is approximately 132 square millimeters or 132,000,000 square microns.

Using the value for transverse axes of cells given in Table II, we may find the average cross-sectional areas of the 1 day epidermal cell.

If the diameter of a 1 day cell = 8.9  $\mu$ , the cross-sectional area =  $\pi r^2 = \pi(4.45)^2 = 62.2$  square microns. The total number of cells on the 1 day seed would be equal to

$$\frac{\text{Surface area of the seed}}{\text{Surface area of the cell}} = \frac{4,190,000}{62.2} = 67,400$$

and the number of cells per square millimeter would be equal to

$$\frac{\text{Total number of cells}}{\text{Surface area of the seed}} = \frac{67,400}{4.19} = 16,100.$$

It would follow that if the number of epidermal cells should remain constant from the first day, the greatly increased surface area of the seed would reduce the total number of cells per square millimeter to the extent of  $67,400/132.34 = 509$ , a number so small that it falls beyond the range of possibility.

These estimates of increase in surface area of the developing seed, as compared with the increase in length of transverse axes of the epidermal cells on the first and twentieth days, show conclusively that cell enlargement will not account for the extension of the epidermal layer of the seed-coat. As a matter of fact, this same idea is supported by a series of Balls' own diagrammatic drawings (2, Fig. 13) which indicate the relative amount of enlargement from the time of fertilization to maturity in both seeds and cells of the epidermal layer of the seed-coat. Measurements of these drawings show an increase in seed size from a length of 1.5 mm. and a width of 1 mm. on the third day from fertilization, to a length of 10 mm. and a diameter of 6 mm. at maturity. Corresponding measurements of cells from the epidermal layers in the same figure at magnifications of 150 indicate relative dimensions of  $1.5 \times 1.5$  mm. at three days and  $4.0 \times 2.0$  mm. at maturity. This amount of cell enlargement alone obviously will not explain the presence of a continuous layer of epidermal cells upon the coat of the much enlarged seed in the varieties used by Balls.

In the mounts of living material the cytoplasmic behavior of epidermal cells not engaged in fiber formation could not be accurately determined. The use of paraffin material became necessary, therefore, and resulted in the finding of large numbers of dividing cells in the epidermal layer and the apparent formation of fibers from daughter cells resulting from these divisions.

#### CELL DIVISIONS IN EPIDERMAL CELLS

For this later study material of each daily stage was fixed in Flemming's Weak solution and also in a mixture of 4 per cent formalin and 70 per cent alcohol. The latter method of fixation gave surprisingly excellent results and the present report is based upon material so treated. The paraffin sections were from  $4 \mu$  to  $15 \mu$  in thickness, were stained with haematoxylin, and counterstained with safranin and gold orange. The appearance of the epidermal cells during the first ten, the thirteenth, and the seventeenth day from flowering is illustrated in Figure 4. In this diagrammatic form no attempt has been made to show cytological details. A few resting nuclei, some nuclei in early stages of division, and one cell in the process of cell plate formation (4 day) are indicated. The sketches are all made to the same scale ( $\times 1500$ ), and illustrate graphically the increase in length of transverse axes given numerically in Table II. In the 1 day group, the nuclei of the end cells were not in median sections, and were resting, the

nuclei of the cells next to these were in early division stages, the nuclei of the next two cells on either side were again resting, and the nucleus of the large central cell was in active division. This particular instance shows regular alternation of resting and dividing cells. That this is not always the situation is shown in the 4 day group where two adjacent cells are in active nuclear division. In the 2 day group the second cell from the right was in

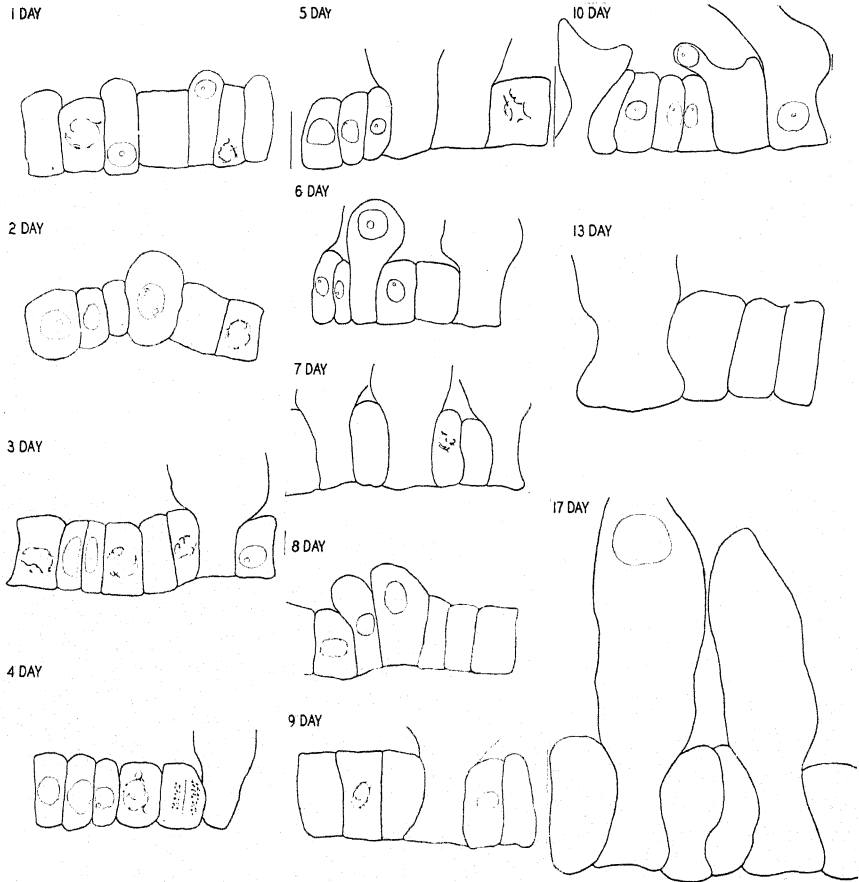


FIGURE 4. Camera lucida sketches from paraffin material of rows of epidermal cells from the seed coat of *Gossypium hirsutum* L. at intervals up to seventeen days from the date of flowering.  $\times 520$ .

the process of division while the two cells to the left of the large central one had apparently recently divided in another plane. The 3 day group shows one fiber cell, three dividing nuclei, and two cells, the second and third from the left, where cell plate formation and nuclear reorganization have

been completed but a short time. In the 4 day sketch three resting cells with nuclei of irregular outline are balanced by two dividing cells and one developing fiber. The 5 day sample shows two fiber cells and one actively dividing cell in contrast to the three resting cells. The 6 day group contains one older fiber cell at the extreme right, a dividing cell next to it, three resting cells, and one young fiber cell. The 7 day, 8 day, and 9 day sketches illustrate no additional points with the exception of the balance which is obtained in cell size through the rapid succession of cell divisions. At 10 days abnormalities, quite uncommon in these early stages, are shown. The two smaller fibers are depressed at the tip, and in the fiber, at the extreme right, the nucleus has remained at the base of the cell instead of taking its usual position at this stage near to the tip. The 13 day group illustrates the progressive increase in cell size. The 17 day sketch shows that initiation of fiber growth has recently taken place.

It was found in all serial sections that were examined that constrictions such as are shown at the base of the fiber at the extreme left of the 10 day sample are the result of the plane at which the section is made. Cutting near to the surface of the fiber in any longitudinal section will give this appearance of constriction at its base. The nearer to the surface the section is made, the greater the amount of attenuation in the basal part. The tendency to enlargement of fiber diameter at the surface line of the epidermis assists in producing this effect. Median sections of these same fibers showed a proportion between base and fiber more nearly like that illustrated in the 5 day group.

This is not in agreement with Barritt's (5) findings upon which he based his theory of fiber nutrition. He says (p. 489), "No development of the base of the hair cells occurs after differentiation. The neighboring epidermal cells, however, develop enormously and subject the former to considerable lateral pressure which, together with the thickening of the wall, closes the lumen at the bases of the hairs at an early age. The boll cavity is regarded as an intercellular space continuous with that of the mesophyll, and the nutrition of the lint hairs like that of the palisade cells is derived from the intercellular liquid."

#### "BOLL-SAP"

An examination of bolls in all stages of development failed to reveal the presence of any liquid or semi-liquid substance in the boll cavity. In Figure 1, it may be observed that by the ninth day the entire boll cavity is filled with developing fibers and that this condition continues on through succeeding stages. The small spaces between the fiber mass and the boll wall seen in some sections are due to crushing in preparation of the sections. Before the ninth day the space between the fiber mass and the boll wall was filled with gaseous, not liquid material. Any liquid which was observed

in the boll cavity was due to exudation of the aqueous cell contents from fibers which had been cut in the process of opening the boll. When this injury was avoided by careful cutting of the boll wall, the entire severed portion could be lifted away and the absence of any liquid substance in the cavity observed.

#### DISCUSSION

Many of the questions which arose during the early study of sections of fresh cotton seeds in different stages of development seem to be adequately answered through the findings in thinner, clearer sections of paraffin material. The large numbers of dividing cells will account for the extension of the epidermal layer in proportion to the increase in surface area of the seed. The formation of fibers from cells which are apparently daughter cells resulting from these divisions is in keeping with the delayed fiber growth initiation which was observed earlier in living material. We may be quite certain that in the variety studied cell division in the epidermal layer takes place quite actively until the eleventh or twelfth day; that it probably continues at a somewhat reduced rate for the next five or six days; and that upon some parts of the seed surface it may even be found after the twentieth day. These latter points will be finally determined only through the study of large numbers of sections at each daily stage from all parts of the seed and cannot be answered from the median sections alone to which this study was limited.

The possibility of nutrition of the developing fiber in any other fashion than through its basal connection with the seed seems to be quite remote. The absence of a liquid substance in the boll cavity which has commonly been designated as "boll-sap," and the lack of evidence of basal attenuation in median sections of fibers seems to be sufficient evidence upon which to base a contention of this idea. The degree of variation in fibers from the same seed, the delicacy of the fibers during early stages of development, and the large number of confusing factors which are introduced in the preparation of the mounts, planes of sectioning, etc., might be expected to furnish some specimens which would verify almost any statement, within reason, concerning the cotton fiber. An examination of the entire range of possibilities in each instance should either substantiate the idea, relegate it to the position of an exception and not a rule, or discount it entirely. More of the existing confusion with respect to the morphological and cytological characteristics of the fiber probably arises from piecemeal observation than from erroneous interpretation. This lack of thoroughness is in some instances almost excusable because of the wide ranges which must be covered in material so difficult to manipulate, and consisting largely of cellulose whose fundamental properties are themselves the basis for much controversial discussion. It seems to be evident, however, that the most

direct method of establishing a basis for classification of mature fibers may be sought through an understanding of their developmental stages with particular emphasis upon the points at which the first permanent differentiation occurs.

#### SUMMARY

1. Observations of the early stages of elongation of fibers from the seed-coat of *Gossypium hirsutum* in mounts of fresh material show wide variation in fiber length.

2. The appearance of the cytoplasm indicated that growth initiation had taken place more recently in the shorter than in the longer fibers.

3. The thirty-two-fold increase in surface area of the seed during the first twenty days, in comparison with the two-fold increase in length of the transverse axes of epidermal cells during the same period, indicates that cell enlargement is not alone responsible for the tangential extension of the epidermal layer of the seed-coat.

4. Large numbers of dividing cells in the epidermal layer from the date of flowering to the twelfth day following, reveal the chief method of extension of the epidermal covering.

5. A slight decrease in number of dividing cells after the twelfth day, and the presence of still fewer after the fifteenth day from flowering, suggest a progressive retardation in cell division which may begin as early as the tenth day. These observations are limited to median cross-sections of the seed and are not representative of the entire surface.

6. Early stages of fiber elongation from epidermal cells which were apparently daughter cells of recent divisions furnished direct evidence that fibers may originate from cells which are not yet formed upon the date of flowering.

7. The nutrition of the fiber in any other fashion than through its basal connection with the seed seems to be quite unlikely. Median sections of the bases of fibers in all stages of development show neither extreme attenuation nor extreme constriction. An examination of bolls in all stages of development failed to reveal the presence of any liquid or semi-liquid substance in the boll cavity.

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## THE EFFECT OF ETHYLENE AND ILLUMINATING GAS ON ROSES

P. W. ZIMMERMAN, A. E. HITCHCOCK, AND W. CROCKER

In a report on illuminating gas injury to greenhouse plants, in 1911 Wilcox (6) stated that rose plants had been defoliated and actively growing shoots had been killed. Since that time seven papers dealing with the effect of gas on roses have been published. Stone (5) in 1913 reported similar injuries to roses in Massachusetts. Crocker (1) in 1928 reported a case of gas injury to polyantha roses in greenhouses where they were being forced for Easter trade. They were plants with many new shoots arising from dormant canes. Figure 2 appearing in his publication shows a normal plant from a greenhouse free from gas and a plant from a greenhouse containing gas in sufficient concentration to cause epinasty and leaf fall. Since the response of roses to gas is much less striking than that of tomatoes, Crocker strongly recommended that test plants such as the tomato be placed in greenhouses whenever the presence of gas is suspected.

Zimmerman, Crocker, and Hitchcock (7, 8), in November 1930, reported that 0.01 per cent (1 to 10,000) illuminating gas caused yellowing along the veins and abscission of rose leaflets and leaves of the plants exposed for 48 hours at room temperature. Flower buds of the treated plants opened prematurely and petals fell earlier than those of control plants. Recovery of treated plants was later characterized by active growth from many of the latent buds, whereas the hand-defoliated control plants produced shoots from a fewer number of buds. No noticeable injury resulted from a seven day treatment with 0.02 per cent of gas when held at 45° to 50° F.

In December 1930, Shull (3) also reported abscission of leaves and petals from roses treated with ethylene and illuminating gas. He used comparatively high concentrations of illuminating gas (0.1 and 0.2 per cent) and ethylene (0.1 and 0.05 per cent) on Red Radiance roses. In addition to petal drop and defoliation, he mentions reduced vigor, though the latter point, he stated, needs more investigation since it has been claimed that ethylene forces dormant buds by stimulating them. His preliminary tests showed that hand-defoliated plants grew branches more vigorously than those of gas-defoliated plants.

In April 1931, Shull (4) again reported on reduced vigor resulting from gas treatment. He used as a basis for his conclusions a hand-defoliated and a gas-defoliated Red Radiance rose plant. Upon recovering, the plant treated with 0.2 per cent illuminating gas regenerated 84 new shoots as compared to 36 for the control plant. Twenty-three days after removal from the gas, the shoots of both plants were cut off, measured, and weighed.



Though the treated plant had many more shoots than the control, the total length of all shoots was about the same for both plants. The control plant produced two grams more green weight than the gassed plants. This he believes brings into question the idea that ethylene is a stimulant. It was further observed that apical dominance was interfered with when plants were treated with gas, disturbing the natural tip to base growth correlations. This unnatural balance was maintained by the plant until all the new growth was removed. Thereafter, it was free from this influence of gas and new shoots came out normally. These results are not in entire agreement with those of the authors of this paper.

The purpose of this paper is to report the typical responses of seven varieties of roses exposed to known concentrations of ethylene or illuminating gas for different time periods at various temperatures.

#### MATERIAL AND METHOD

The majority of rose plants used in these experiments were grafted hybrid teas (Briarcliff, Templar, Double White Killarney, Madame Butterfly, Mrs. Calvin Coolidge, Souvenir Claudius de Pernet, Red Radiance) obtained from A. N. Pierson of Cromwell, Connecticut or from Jackson and Perkins, Newark, New York. The plants were grown in large pots or jars containing well fertilized soil. They were treated with gas in various stages of growth up to maturity. Usually any one plant at the time of treatment had shoots of different stages of maturity so that observations could be made of the effect of gas on different aged tissues under the same conditions. Cut buds and flowers came either from the Boyce Thompson Institute rose garden or from the experimental plants in the Institute greenhouses.

The illuminating gas which was used came from the mains of the Westchester Lighting Company at Yonkers, New York. The analysis indicated 5.6 per cent illuminants. It was estimated that at least 50 per cent of the illuminants was ethylene. On this basis approximately 3 per cent of the illuminating gas was ethylene.

The pure ethylene employed in the experiments was purchased from the United States Industrial Chemical Company, New York City. Analyses of the gas showed the following absorption:

	Per cent
Potassium hydroxide.....	0.3
Potassium pyrogallol.....	0.5
Saturated solution of bromine.....	97.2
	<hr/>
Total.....	98.0

It was assumed that the remaining 2 per cent was nitrogen and that approximately 97 per cent of the gas was ethylene.

Plants were exposed to gas in large Wardian cases or 20-gallon cans. Two cases were 3 ft.  $\times$  3 ft.  $\times$  7 ft. and two were 4 ft.  $\times$  4 ft.  $\times$  5 ft. These were equipped so that the desired quantity of gas could be admitted through small openings. This was sometimes done but generally the gas was measured in glass cylinders, which were set with the plants in the cages, the stoppers were removed and the door quickly closed and sealed with plasteline. Similarly the lids were sealed on the 20-gallon cans. If the experiment lasted more than 48 hours, the cases were opened, fanned out, and re-treated as at first. The glass cases were considered superior to the cans because the plants were exposed to light during the treatment. This is particularly important when the period of exposure is more than 48 hours. During the summer the cases were outside on stands and shaded by means of slats or muslin to prevent the temperature inside the case from rising too high while the sun was shining. The rest of the year the cases were kept in the greenhouses and when necessary, shade was provided.

Table I shows the number of experiments performed, the gas concentrations, and the number of plants used. Other details concerning methods are given under the heading of "Results."

## RESULTS

*Epinasty.* Leaflets of immature leaves near the tips of active shoots showed, when treated, an epinastic growth which caused a slight twisting or curling. While in this condition they are somewhat similar to young partially unfolded leaflets (Fig. 1 A and B). The young leaflets on controls showed a curvature of the petiole and the midrib only, whereas the treated leaflets showed additional bending and curling of the blades. Leaves are most sensitive when they are unfolding to assume their spreading position (Fig. 1 A). Epinasty occurred within a 24-hour period when at room temperature. Plants removed from strong ethylene (1 to 25,000) recovered from all signs of epinasty within a day. Recovery was slow when the time of exposure was long enough to cause abscission of some of the leaves. The leaf shown in Figure 1 A recovered after several days and the bud developed into a flower after ten days.

Mature leaves did not make epinastic growth under the conditions to which they were subjected.

*Abscission.* Roses commonly lost some of their leaves when exposed to gas, depending upon the concentration, the time of exposure, and the temperature. A 24-hour treatment at 70° F. with ethylene at 1 to 25,000 caused no abscission, whereas a 48-hour treatment with this concentration caused 50 per cent of the leaves to fall and after 96 hours, complete defoliation (Fig. 2 A). With this concentration at 41° F. no abscission resulted from a 7-day treatment (Fig. 2 B). Likewise at a constant temperature of

50° F. no injury occurred after eight days in 1 to 5000 illuminating gas (equivalent of 1 to 165,000 ethylene). At 60° F., however, a White Killarney rose plant treated for seven days with ethylene (1 to 100,000) lost 72 leaflets and 21 of its 37 leaves; at 70° F. in ethylene (1 to 100,000) for the 7-day period a Killarney plant lost 37 leaflets and 30 of its 34 leaves. This shows a relationship between temperature and abscission of leaves when the gas is in the same concentration. Somewhere between 50° and

TABLE I

CONCENTRATIONS OF ILLUMINATING GAS USED, THE NUMBER OF TESTS MADE, AND THE NUMBER OF PLANTS TREATED. THE TESTS WITH CUT ROSE BUDS ARE NOT HERE INCLUDED

Kind of gas	Concentration given as 1 part of gas to that of air	Length of treatment in hours	Number of tests made with a given concentration	Number of plants used
Ethylene	1 to 800	72	1	2
	1 to 25,000	24, 48, 72, 96, 168	10	12
	1 to 30,000	96	2	15
	1 to 60,000	72	1	8
	1 to 100,000	24, 48, 72, 96	8	14
	1 to 1,000,000	48, 168	2	11
	1 to 3,000,000	48, 96, 120, 168	4	40
Illuminating gas	1 to 25	72	1	2
	1 to 50	62	1	2
	1 to 100	62, 44	2	7
	1 to 200	48, 64	2	4
	1 to 500	72	1	2
	1 to 1000	75	2	6
	1 to 2000	75, 72, 96	5	12
	1 to 5000	72, 48, 192	5	17
	1 to 6000	48	1	2
	1 to 7000	48	1	4
	1 to 8000	72	1	6
	1 to 9000	72	1	6
	1 to 10,000	48, 67, 72, 113	7	26
	1 to 12,000	72, 113	2	10
	1 to 14,000	72	1	4
	1 to 20,000	72, 96, 135	4	8
	1 to 40,000	72, 96, 135	4	8
	1 to 75,000	72	2	4
	1 to 100,000	30 days	1	16
Total			72	248

60° F. lies the critical temperature which might be called the division point above which gas could cause rose leaves to absciss.

In comparing the effect of high and low concentrations at room temperature it was found that 1 to 25,000 ethylene causes approximately 50 per cent abscission in less than two days, whereas abscission just began

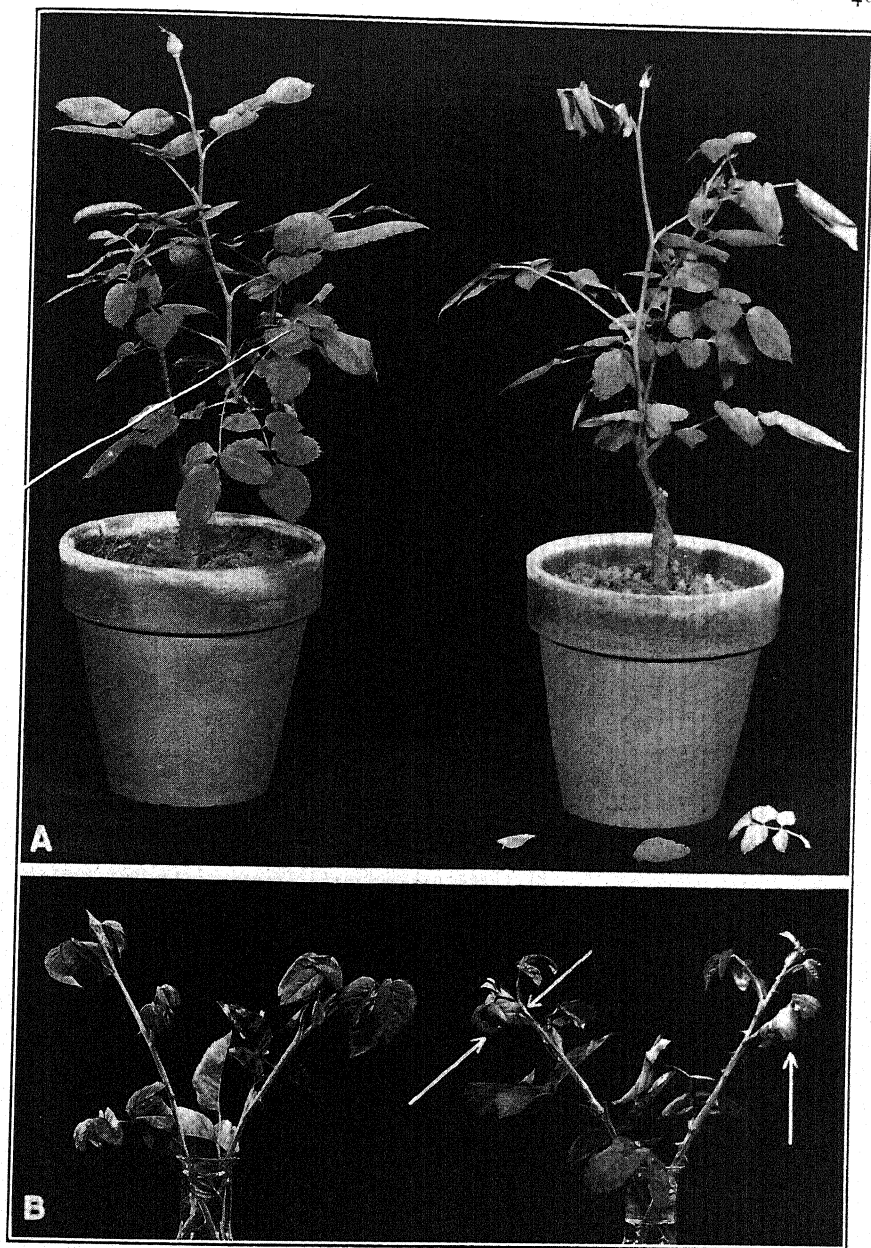


FIGURE 1. (A) At right, Briarcliff rose plant showing epinasty of young leaves after 72 hours in 1:10,000 illuminating gas and the fall of an entire leaf and two leaflets. Control plant at left. (B) At right, similarity of epinasty in young leaves exposed to 1:1,000,000 ethylene for 48 hours. Control shoots at left. In each flask a Butterfly shoot is on the left and a Double White Killarney shoot on the right. White arrows indicate leaves that show curling typical of ethylene or illuminating gas treatment.

after five days in 1 to 3,000,000 ethylene. In 1 to 100,000 ethylene the following abscission of leaves occurred:

(1) None.....	in 24 hours
(2) 19 of 35 leaves.....	" 48 "
(3) 25 of 39 leaves.....	" 72 "
(4) 27 of 33 leaves.....	" 96 "

The leaves still clinging after the 96-hour exposure fell shortly after the treatment was stopped.

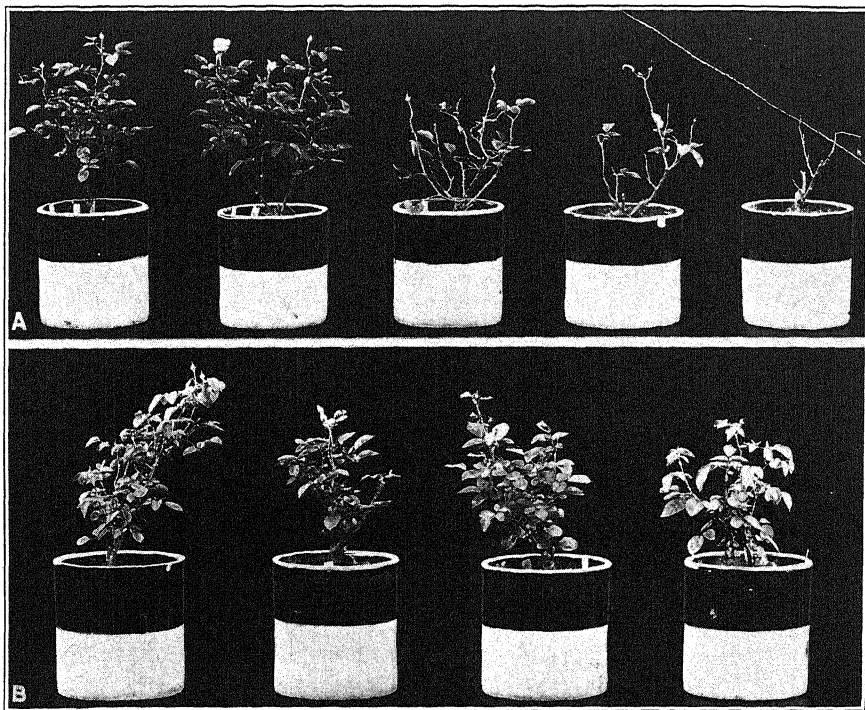


FIGURE 2. Effect of temperature on the response of the Double White Killarney rose to 1:25,000 ethylene. (A) Control plant at left (96 hrs. in air) and four treated plants exposed to ethylene for different time periods (24, 48, 72, and 96 hrs.) at outside summer temperatures. The plant exposed to ethylene for 24 hours showed no injury whereas those exposed for 48 hours or longer showed marked injury. (B) Control plant at left (7 days in air at 40° F.) and three treated plants exposed for different time periods (2, 4, and 7 days) at 40° F. None of the plants exposed to ethylene at 40° F. were injured.

The first leaves to absciss were the oldest on the plants. With continued treatment more of the old leaves and the basal leaves of new shoots fell. Finally the youngest leaves and what has been designated middle-aged

leaves were the last to absciss, the latter being the most resistant (Figs. 2 A and 8).

Leaves of the rose have potential abscission layers at the base of the main petiole and at the base of the leaflets. When treated with gas these layers become active and whole leaves or leaflets may be severed from the plant. As a rule more whole leaves than leaflets absciss from the plant.

*Color change in leaves.* One of the most characteristic effects of gas on rose plants was the type of yellowing which it caused on the leaves. After five days in a low concentration of ethylene (1 to 1,000,000) leaves developed a yellow color which followed along the veins (Fig. 3). The first sign of this discoloration appeared at the base of the midrib, and then extended up toward the tip and out into the secondary veins. If the exposure

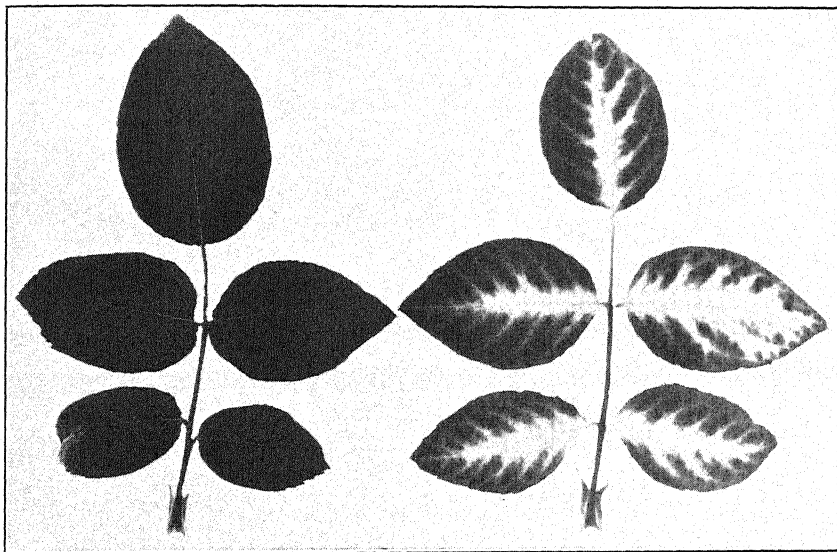


FIGURE 3. At right, leaves of *Rosa* (hybrid tea) var. Madame Butterfly showing the yellowing of the veins of leaflets 3 days after removal from a 48-hour treatment in 1:5,000 illuminating gas. Control leaf at left.

to gas was long enough, this discoloration continued until the whole leaflet was yellow. The old leaves showed the characteristic yellowing more readily than young ones. The youngest leaves often showed only slight indications of yellowing along the veins, particularly in high concentrations of gas which caused early abscission. Frequently browning followed yellowing along the veins in the same manner. When plants were treated with high concentrations of gas so that the leaves were caused to absciss while still green, the same color changes appeared after the leaves had fallen, provided they were kept in a high enough humidity to prevent drying.

Some varietal differences appeared in respect to yellowing. *Souvenir Claudius de Pernet*, for example, under some conditions, showed browning instead of yellowing along the veins. At times, however, it went through all the stages described above. Of all the varieties tested, *Madame Butterfly* showed the discoloration of the leaves most readily.

Low temperature prevented coloring of leaves in gas just as it prevented abscission. At 60° F. some yellowing occurred but it was not so pronounced as at higher temperatures.

*Flowers and flower buds.* Cut rose buds that would normally have opened within 24 hours showed two specific responses to ethylene and illuminating gas: first, rapid opening of the bud, and second, early petal fall (Tables II and III and Fig. 4). The gas did not change the color of the petals. Immature rose buds showed additional responses. For example, varieties with pointed buds, such as *Madame Butterfly*, *Briarcliff*, *Souvenir Claudius de Pernet*, and *Templar*, opened with small petals and failed to develop the color characteristic for the type. Varieties like *Radiance*, having the outer petals overlapping the inner ones at the top, abscised from the receptacle before the bud opened. This constitutes petal fall as with other types, the difference being that all the petals clung together as they were in the bud. The abscission of petals as illustrated in Figure 4 occurred earlier for *Madame Butterfly* than for *Briarcliff*. The variety most resistant to gas was *Ellen Wilmott*, in which the petals remained on for six days in 1 to 10,000 illuminating gas. Throughout this period no difference between the treated and control was noted.

The response of cut rose buds was approximately the same over a wide range of concentrations of illuminating gas (Table II) and ethylene (Table III). There was, however, a variation in response at different temperatures (Table III). Buds in 32° F. to 50° F. remained practically like the controls and in this respect the results agree with those showing the effectiveness of low temperature in preventing gas injury to leaves.

Flower buds on rose plants exposed to gas went through approximately the same stages as cut buds. One difference was that very immature buds on plants opened more readily than cut buds. In such cases the petals were very small and lacked the normal pigments. The wide variations in response of different aged rose buds exposed to gas make difficult an accurate statement to cover the entire range of possibilities. Frequently some of the buds on a plant were killed while others were not injured. Figure 1 A shows a plant treated for 72 hours in 1 to 10,000 illuminating gas and though most of the leaves fell, the flower bud persisted and produced a flower 10 days later. There have been cases where the plants were entirely defoliated by gas and yet some of the buds developed into flowers. Buds that were approaching maturity were resistant to high concentrations but younger buds were often injured. The first signs of injury were loss of chlorophyll

TABLE II  
THE EFFECT OF ILLUMINATING GAS ON CUT ROSE BUDS AND FLOWERS

Variety and number of rose buds	Concentration of gas	Results after various hours of exposure			
		24	48	72	96
12 of mixed types	Control	All open		All open, no petal fall	
	1 to 25	All open, nearly all petals fallen	All petals fallen		
3 Briarcliff 2 Butterfly 1 Coolidge 1 Templar	Control	Beginning to open			No petal fall
	1 to 100	All open	All petals fallen		
12 Red Radiance of various stages of maturity	Control	Open, no petal fall	Open, no petal fall	2 petals fallen	
	1 to 1000	6 open, some petal fall; 6 other younger buds not opened	All petals of 6 fallen; 6 others abscissed as buds from receptacle		
12 Red Radiance of various stages of maturity	Control	Buds open, no petal fall	No petal fall	No petal fall	No petal fall
	1 to 5000	9 open, many petals fallen; 3 buds not open	All petals of 9 fallen; 3 buds abscissed petals before they unfolded		
6 of mixed varieties	Control	All open		All open, no petal fall	
	1 to 5000	All open, many petals fallen			
	1 to 10,000	All open, some petals fallen	All petals fallen		
6 Red Radiance	Control			All open, no petal fall	
	1 to 8000			4 open, petals fallen, 2 abscissed without opening	
12 Red Radiance of various stages of maturity	Control			All open, no petal fall	
	1 to 10,000	All open, all petals fallen			
12 Red Radiance, Red Star, and several unnamed varieties	Control	All open, no petal fall	All open, no petal fall	All open, no petal fall	
	1 to 14,000	All open		All petals fallen	



and yellowing of the peduncle. This was followed by browning until the injured bud shriveled and finally fell from the plant. The peduncles frequently abscised sharply from the stem but in very immature shoots there was no sharp line of demarcation, a portion of the stem being killed back.

TABLE III  
THE EFFECT OF ETHYLENE ON CUT ROSE BUDS AND FLOWERS\*

Variety and number of rose buds	Concentration of gas	Results after various hours of exposure			
		24	48	72	96
6 (Briarcliff, Butterfly, Coolidge, Templar)	Control	All open, no petal fall			
	1 to 100,000	All open, Briarcliff no petal fall; Butterfly, Coolidge and Templar all petals fallen			
10 Briarcliff of varying stages of maturity (see Fig. 4)	Control	All open	No petal fall	No petal fall	No petal fall
	1 to 100,000	2 immature buds retarded in opening; 8 buds open, all petals fallen from 4; 4 open, no petal fall	All petals of 6 fallen; nearly all petals fallen of 4	The very immature buds open with petals much smaller than controls (see Fig. 4)	All petals fallen
10 Butterfly of varying stages of maturity	Control	All open			No petal fall
	1 to 100,000	All open, nearly all petals fallen			
2 Red Radiance at each temperature indicated	Controls at 32°, 40°, 50°, and 70° F.	Those at 70° open		No petal fall, buds at 32° F. not fully open, some petal fall from the 70° buds 4 hrs. after experiment ended	
	1 to 100,000 at 32°, 40°, 50°, and 70° F.	At 70° all petals abscised, no petal fall at other temperatures		At 40° and 50° no petal fall until 4 hrs. after removal from gas and placed at room temp., buds at 32° appeared like the controls	

\* All exposures were made at room temperature except as indicated for the last two entries under treatment in which four different constant temperatures were used.

This is illustrated in Figure 8 by the lowest shoot on the left of the treated plant.

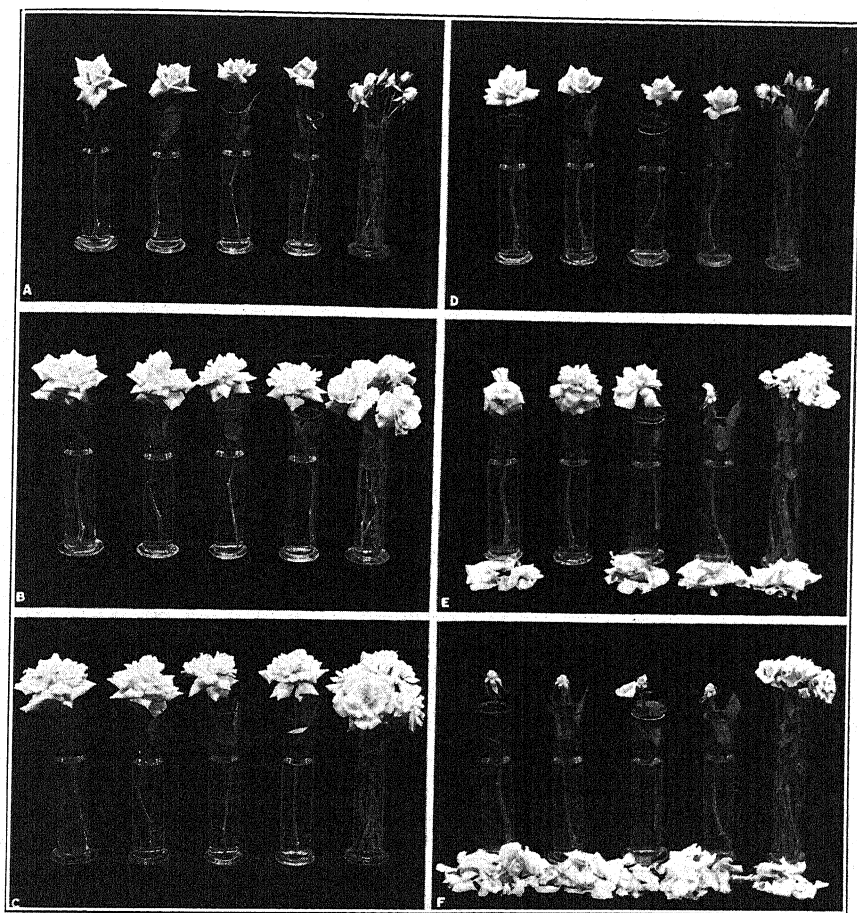


FIGURE 4. Effect of ethylene (1:100,000) on the buds of Briarcliff rose at different stages of development. Control lots at left as they appeared (A) at the beginning, (B) 24 hours, and (C) 48 hours later. Treated lots at right as they appeared (D) before treatment, (E) 24 hours, and (F) 48 hours later. Ethylene hastened the opening of all buds except those that were very small. The increase in size of petals of small buds was retarded by the ethylene treatment.

*Retardation of growth.* In relatively high concentrations of gas growth is retarded or inhibited in actively growing regions. Such a condition may be regarded as a state of rigor since growth is resumed after the plants are removed from gas. The stunting effect of gas on young shoots is shown in Tables IV and V. The values indicate considerable irregularity, but they

show that growth is definitely retarded and that the stronger the gas the greater the retardation. These measurements show the growth after long periods of treatment. Other data not recorded in the table show that most of this growth occurred during the first 48 hours, and that thereafter the stems were nearly at a standstill. This is in accord with effects of ethylene on seedlings (2).

TABLE IV  
THE RETARDING EFFECT OF ILLUMINATING GAS ON YOUNG ROSE SHOOTS

Variety	Concentration of gas	Period of treatment in days	Original length of shoot in inches	Length of shoots at end of treatment in inches	Amount of elongation during treatment in inches
Briarcliff	Control 1 to 500	3	8.5	10.0	1.5
		3	12.0	12.5	0.5
"	Control 1 to 10,000	7	8.25	10.75	2.5
		7	9.25	9.25	0
Coolidge	Control 1 to 40,000	7	11.25	13.5	2.25
		7	12.5	14.0	1.5
"	Control 1 to 10,000 1 to 20,000	3	17.25	18.75	1.5
		3	13.5	13.5	0
		3	16.25	16.25	0
Madame Butterfly	Control	7	10.0	14.75	4.75
	"	7	7.0	11.0	4.0
	1 to 20,000	7	9.25	10.25	1.0
	"	7	11.75	12.5	0.75
	1 to 40,000	7	7.25	7.5	0.25
	1 to 40,000	7	6.25	7.75	1.5
"	Control	3	15.0	17.0	2.0
	1 to 20,000	3	19.0	19.75	0.75
	1 to 10,000	3	12.25	12.5	0.25
	1 to 1000	3	14.25	14.5	0.25
Templar	Control	7	11.75	14.75	3.0
	1 to 40,000	7	13.25	13.5	0.25
"	Control	3	19.5	21.0	1.5
	1 to 20,000	3	13.0	13.25	0.25
	1 to 10,000	3	6.75	7.0	0.25
	1 to 10,000	3	6.5	6.5	0
	1 to 10,000	3	10.0	10.0	0
	1 to 10,000	3	11.75	12.0	0.25

*The forcing effect of ethylene on latent buds.* In the earlier experiments, it appeared that rose plants recovering from illuminating gas injury produced more new shoots than the controls. Figure 8 shows a plant which, after having been exposed to gas, regenerated new growth from practically all of the latent buds. Shoots arose even in the axils of leaves which still

TABLE V  
THE RETARDING EFFECT OF ETHYLENE ON YOUNG ROSE SHOOTS

Variety	Concentration of gas	Period of treatment in days	Original length of shoots in inches	Length of shoots at end of treatment in inches	Amount of elongation during treatment in inches
Briarcliff	Controls	7	5.5	7.75	2.25
		7	10.5	16.25	5.75
		7	8.25	11.0	2.75
	1 to 1,000,000 ethylene	7	8.25	9.0	0.75
		7	6.5	7.5	1.0
		7	1.75	1.75	0
		7	13.0	13.0	0
Madame Butterfly	Controls	7	11.0	15.0	4.0
		7	7.25	9.0	1.75
		7	5.75	6.5	0.75
		7	5.0	6.25	1.25
	1 to 1,000,000 ethylene	7	6.5	6.75	0.25
		7	10.75	11.5	0.75
		7	5.75	6.0	0.25
		7	8.0	8.25	0.25
		7	6.5	7.25	0.75
		7	8.0	9.0	1.0
		7	1.5	1.5	0
		7	4.25	4.25	0
Killarney	1 to 1,000,000 ethylene	7	3.75	4.0	0.25
		7	8.5	9.0	0.5
		7	3.75	4.0	0.25
		7	7.5	8.0	0.5
		7	7.0	7.0	0
Claudius de Pernet	Controls	7	8.0	12.5	4.5
		7	9.5	11.5	2.0
		7	4.25	7.0	2.75
		7	7.0	8.75	1.75
		7	9.0	10.5	1.5
	1 to 1,000,000 ethylene	7	9.75	10.0	0.25
		7	9.0	9.75	0.75
		7	9.5	9.5	0
		7	1.5	1.5	0
		7	4.0	4.25	0.25
Briarcliff	Control	5	5.75	8.0	2.25
	1 to 3,000,000 ethylene	5	4.0	4.5	0.5
		5	3.0	3.25	0.25
		5	2.75	3.25	0.5
		5	3.25	4.0	0.75
		4	2.75	4.0	1.25
Claudius de Pernet	Control	5	3.5	4.25	0.75
	1 to 3,000,000 ethylene	5	5.5	6.25	0.75
		5	2.0	2.25	0.25
Madame Butterfly	Controls	5	4.0	5.0	1.0
		5	5.0	5.75	0.75
		5	2.75	4.0	1.25
		4	3.25	5.0	1.75
		5	5.75	8.5	2.75
	1 to 3,000,000 ethylene	5	4.0	4.25	0.25
		5	3.25	3.5	0.25
		5	2.75	2.75	0

remained on the plant. This was thought to be contrary to the normal habit of growth.

In order to obtain accurate data on the possible forcing effect of ethylene on roses, 20 plants were pruned so as to leave only one long stem per plant and these were then hand-defoliated before treatment (Figs. 5 A and 6 A). Nine of the plants were used as controls and eleven were treated with ethylene, after which they were placed side by side on a greenhouse bench and allowed to regenerate new shoots. Figures 5 and 6 show how the plants appeared at three different stages of growth. Tables VI to IX show in detail the comparative results. While there were some individual variations, taken as a whole 70 per cent of the treated buds produced new shoots as compared with 44 per cent for the controls. On the average there were 62 per cent more length of shoots and 33 per cent more dry weight in the treated than in the control plants. The first signs of growth appeared on the control plants during the time that the treated ones were being exposed to the gas. Though the ethylene forces buds into activity, apparently there was no growth while the plants were actually in the gas. The earliest growth in the controls usually came near the tips of the stems whereas it is distributed generally over the stems of treated plants (Figs. 5 A and 6 A). Figure 7 shows graphically the average length of shoots as distributed from tip to base along the stems.

These data are not in accord with those of Shull (4). He reported that his treated plant regenerated 84 new shoots as compared to 36 for the control plant. It is not stated how many latent buds were on the plants at the beginning, but assuming both plants had the same number of buds the gas caused 2.25 times the normal number of buds to grow. This ratio is about 1.4 times greater than that reported in Table IX. All of the 84 shoots produced by Shull's treated plant gave approximately the same total length as 36 shoots of his control, but the fresh weight of the control was 2 grams greater than for the treated. In Table VI of the present paper there are two cases where the control showed more dry weight than the treated plants, yet the average for all the plants is shown as 33 per cent in favor of the treated plants.

The average fresh weight produced by 9 check plants is 12.2 grams, and of 11 treated plants 16.1 grams. An examination of the data by Student's method discloses that the odds are about 20 to 1 that the treated plants exceed the check in amount of new growth. Although the average of the treated plants exceeds the average of the checks by 30 per cent the individual variation among the plants results in rather low odds. More plants would be required to increase the odds beyond the figure found.<sup>1</sup>

<sup>1</sup> Statistical data were furnished by Dr. W. J. Youden.

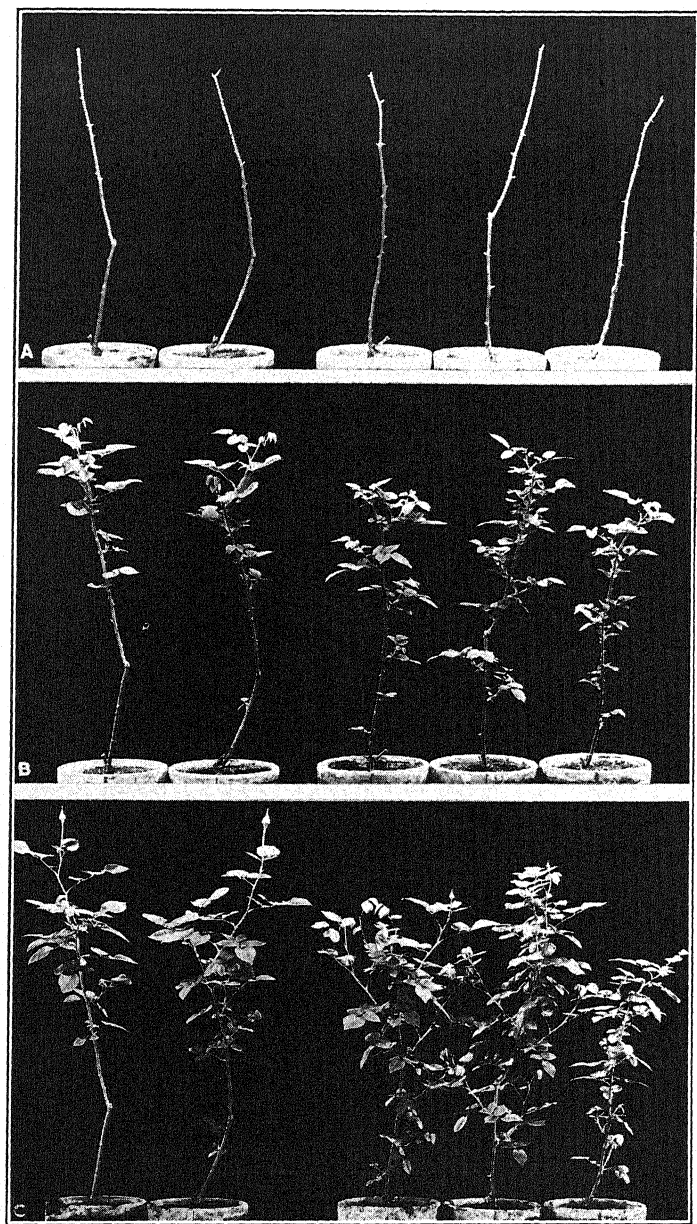


FIGURE 5. *Rosa* (hybrid tea) var. Madame Butterfly showing the growth of shoots at three different stages after removal from a 3-day treatment in 1:60,000 ethylene; two control plants at left and three treated plants at right. (A) Hand-defoliated plants 3 days after treatment, (B) the same plants 16 days, and (C) 32 days after removal from gas.

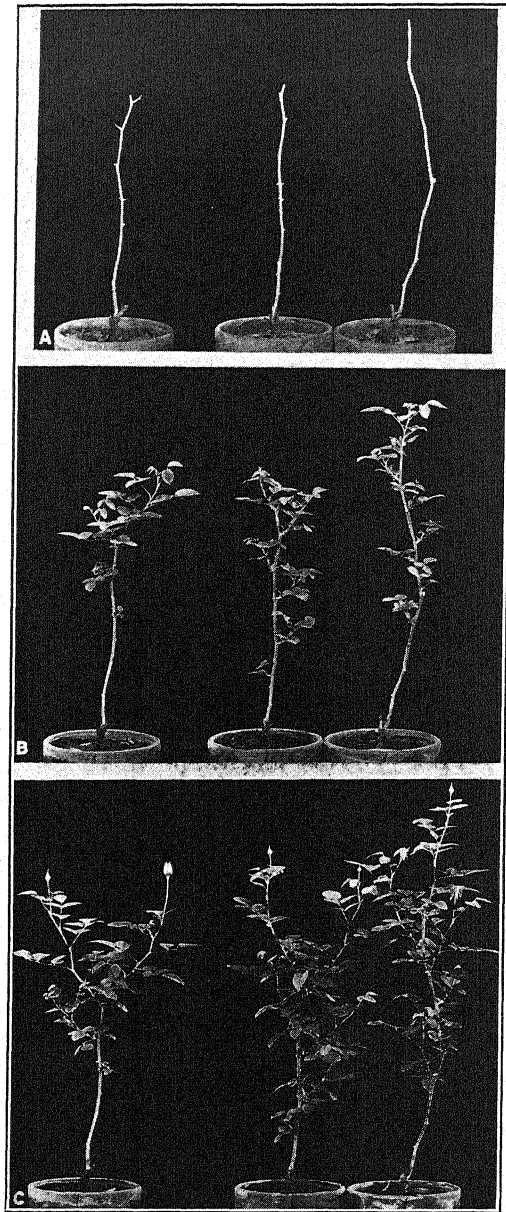


FIGURE 6. *Rosa* (hybrid tea) var. Mrs. Calvin Coolidge showing the growth of shoots at three different stages after removal from a 3-day treatment in 1:60,000 ethylene; one control plant at left and two treated plants at right. (A) Hand-defoliated plants 3 days after treatment, (B) the same plants 16 days, and (C) 32 days after removal from gas.

TABLE VI  
FORCING EFFECT OF ETHYLENE GAS ON THE LATENT BUDS OF THE MRS. CALVIN COOLIDGE ROSE†

Treatment	Total No. buds on the plant	Total No. buds producing new shoots after treatment	Percentage buds producing shoots	Length of each shoot in order from tip down in inches	Total length of shoots in inches	Fresh weight in g.	Total dry weight in g.
Control	11	4	36	10.00* 4.00 1.50 0.50	16.00	16.4	4.57
Control	11	6	55	10.00* 4.75 7.00 1.50 1.00 0.25	24.50	16.8	4.67
1 to 60,000	13	10	77	3.25 10.50* 9.50* 7.50* 5.50 0.12 0.25 8.00* 7.00* 0.25	51.87	27.1	7.76
1 to 60,000	10	8	80	2.75 4.00 9.25* 11.00* 11.00* 4.25 1.50 0.25	44.00	21.5	5.81
1 to 60,000	12	8	66	1.50 1.50 1.00 5.50 3.00* 1.25 1.10 3.75	18.60	11.6	2.96
Control	15	5	33	7.25* 2.25 2.75 1.75 9.75*	23.75	17.0	5.12

† Plants were pruned so that only one long shoot remained and this was hand-defoliated before treatment. Plants were divided into lots; one lot was placed in a case with air, and another lot in a case with ethylene gas from April 24th to April 27th inclusive. The plants were then allowed to grow in the greenhouse until June 1st at which time measurements were taken and shoots removed and weighed.

\* Denotes shoots having flower buds.



TABLE VI (Continued).

Treatment	Total No. buds on the plant	Total No. buds producing new shoots after treatment	Percentage buds producing shoots	Length of each shoot in order from tip down in inches	Total length of shoots in inches	Fresh weight in g.	Total dry weight in g.
1 to 30,000	10	8	80	2.38 1.00 1.68 1.12 0.38 3.00 1.25 0.50	11.31	11.9	3.89
Control	19	7	37	0.12 0.12 0.50 2.00 0.50 7.50 0.25	10.99	9.6	2.99
1 to 30,000	12	7	58	0.25 0.50 0.50 0.50 3.00 3.00 2.50	10.25	7.7	2.31

TABLE VII  
FORCING EFFECT OF ETHYLENE GAS ON THE LATENT BUDS OF MADAME  
BUTTERFLY ROSE PLANTS†

Treatment	Total No. buds on the plant	Total No. buds producing new shoots after treatment	Percentage buds producing shoots	Length of each shoot in order from tip down in inches	Total length of shoots in inches	Fresh weight in g.	Total dry weight in g.
Control	17	6	35	9.25* 0.88 0.25 1.12 1.00 0.25	12.75	8.0	2.43
Control	12	8	66	1.50 1.25 1.00 0.50 2.75 3.38* 4.00* 1.00	15.38	11.7	3.37

† Plants were pruned so that only one long shoot remained and this was hand-defoliated before treatment. Plants were divided into lots; one lot was placed in a case with air, and another lot in a case with ethylene gas from April 9th to April 13th inclusive. The plants were then allowed to grow in the greenhouse until June 1st at which time measurements were taken and shoots removed and weighed.

\* Denotes shoots having flower buds.

TABLE VII (Continued)

Treatment	Total No. buds on the plant	Total No. buds producing new shoots after treatment	Percentage buds producing shoots	Length of each shoot in order from tip down in inches	Total length of shoots in inches	Fresh weight in g.	Total dry weight in g.
1 to 30,000	18	11	61	2.00 1.88 1.25 1.00 0.88 0.12 0.38 1.25 2.75 1.38 5.00*	17.89	13.3	4.33
1 to 30,000	10	9	90	9.00* 0.25 1.88 1.88 3.38 1.50 1.38 0.12 1.00	20.39	14.7	4.43
Control	13	7	54	0.12 0.25 3.12 7.25 2.50 0.50 0.12	13.86	8.8	2.48
1 to 30,000	13	7	54	0.12 1.75 2.12 2.00 4.50* 2.12 12.50*	25.11	17.3	5.73
Control	8	4	50	9.75* 10.75* 3.75 0.25	24.50	13.0	3.51
1 to 60,000	8	6	75	10.75* 12.00* 5.50 4.50 3.00 0.25	36.00	20.3	5.34
1 to 60,000	11	8	73	11.75* 10.75* 8.75* 1.00 1.50 0.50 0.25 7.25*	41.75	21.4	5.93

TABLE VIII  
FORCING EFFECT OF ETHYLENE GAS ON THE LATENT BUDS OF TEMPLAR  
ROSE PLANTS\*

Treatment	Total No. buds on the plant	Total No. buds producing new shoots after treatment	Percentage buds producing shoots	Length of each shoot in order from tip down in inches	Total length of shoots in inches	Fresh weight in g.	Total dry weight in g.
Control	15	6	40	0.37 0.25 1.25 1.00 1.87 0.25	4.99	8.5	2.63
1 to 30,000	10	7	70	1.50 2.50 2.75 3.12 0.25 0.12 0.75	10.99	10.0	3.28

\* Plants were pruned so that only one long shoot remained and this was hand-defoliated before treatment. Plants were divided into lots; one lot was placed in a case with air, and another lot in a case with ethylene gas from April 9th to April 13th inclusive. The plants were then allowed to grow in the greenhouse until June 1st at which time measurements were taken and shoots removed and weighed.

TABLE IX  
SUMMARY OF DATA APPEARING IN TABLES VI, VII, AND VIII SHOWING THE FORCING EFFECT  
OF ETHYLENE ON LATENT BUDS OF ROSE PLANTS THAT WERE HAND-DE-  
FOLIATED BEFORE TREATMENT

	Nine control plants	Basis of nine treated plants	Eleven treated plants	Per cent gain or loss over control plants
Total number buds on all plants	121	104	127	
Percentage buds producing new shoots	44	70	70	+26
Total length shoots for all plants in inches	147	236	288	+62
Total fresh weight all shoots in grams	109.8	145	176.8	+33
Total dry weight all shoots in grams	31.8	42.4	51.8	+33
Average length shoots per plant in inches	16.3	26.2	26.2	+62
Average fresh weight of shoots per plant in grams	12.2	16.1	16.1	+32
Average dry weight of shoots per plant in grams	3.53	4.71	—	+33

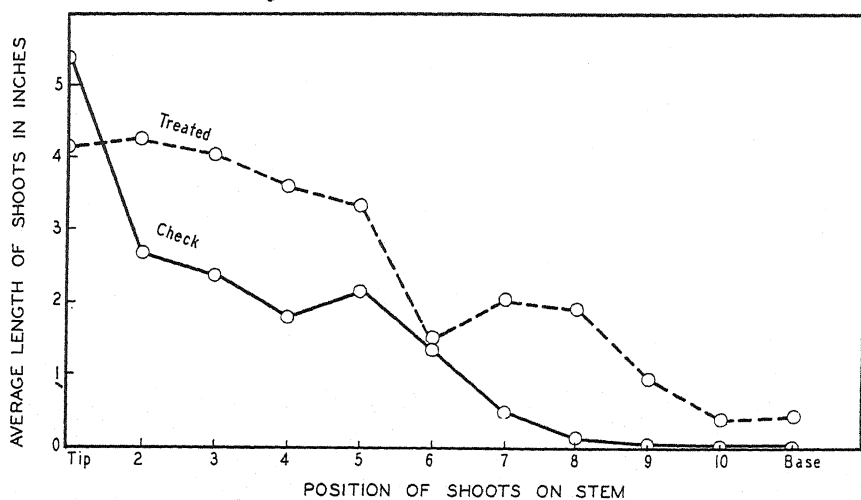


FIGURE 7. Average length of new shoots in inches for each position from tip to base on the main stem. All plants were hand-defoliated before treatment began. Values appearing in this graph are averages of those in Tables VI to VIII.



FIGURE 8. At right, *Rosa* (hybrid tea) var. *Madame Butterfly* showing recovery 10 days after removal from a 3-day treatment in 1:2,000 illuminating gas. Arrows indicate new shoots growing from axils of old leaves. Many other new shoots are growing where leaves have fallen. Control plant at left.

Shull further states that apical dominance was interfered with when plants were exposed to gas, disturbing the natural tip to base correlations. An examination of the data in Tables VI to VIII discloses that five of the nine controls had the longest shoot at the middle or near the base of the shoot producing region. The other four had the longest shoots definitely at the tips. Four of the eleven treated plants had the longest shoots definitely at the tip. The odds are about the same for controls and treated plants. Here again a larger number of plants would be required to settle this question of apical dominance.

#### SUMMARY

1. Ethylene and illuminating gas caused epinasty of young rose leaves. It was noted that this epinastic growth as a rule was not particularly striking and might readily be confused with the natural drooping position of normal young leaves. Old leaves did not show epinasty when treated with gas. Epinasty occurred in concentrations with an ethylene equivalent ranging from 1 to 25,000 to 1 to 3,000,000.
2. Abscission of leaves varied with (a) the age of the leaf, (b) the concentration of the gas, (c) the temperature, (d) the time of exposure, and (e) the variety of rose. The oldest leaves were the first to abscise and the middle-aged leaves were the most persistent, in some cases remaining on the plant and active after removal from the gas. All concentrations of ethylene used (1 to 25,000 to 1 to 3,000,000) caused abscission, the time required varying from less than 48 hours for the strong to 120 hours for the weak gas. No abscission occurred when the temperature was 50° F. or lower. Butterfly appeared more sensitive than the other varieties and Pernet was the most resistant.
3. Gas caused yellowing along the veins beginning first at the base of the midrib and extending out into the secondary veins until the whole leaf was yellow. High concentrations of gas caused abscission of leaves before discoloration, but such leaves still yellowed if not allowed to dry. Frequently browning followed yellowing along the veins in the same order. Discoloration was influenced by the concentration of the gas, age of the leaf, variety of the rose, humidity, and temperature.
4. Cut flower buds approaching maturity opened when exposed to gas and lost most of their petals within 24 hours, varying somewhat according to variety. The gas did not change the color of the flower.
5. Mature flower buds on rose bushes responded to gas approximately as described for cut flowers. Younger buds opened prematurely, in which case the flowers were abnormally small and lacked the color characteristic for the variety.
6. Gas interfered with elongation of young shoots so that after 48 hours

there was practically no growth. If injury was not too severe, growth was again resumed after removal of the plant from the gas.

7. Ethylene caused an abnormally large number of latent rose buds to produce shoots, 70 per cent of all buds producing shoots on the treated plants compared with 44 per cent for the controls. The treated plants exceeded the controls in shoot length by 62 per cent and in dry weight of new shoots by 33 per cent.

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11

## FREEZING POINT DEPRESSIONS OF ASPARAGUS SHOOTS DETERMINED BY A THERMO-ELECTRIC METHOD

EVELYN I. FERNALD

In an earlier study (5) of the sap of different stems it was found in the majority of cases that the upper 18 centimeter portions of actively growing stems had a greater depression of the freezing point than successive lower portions. In that work the determinations were made by the Beckmann method on sap extracted from previously frozen tissue. In this study of shoots of asparagus (*Asparagus officinalis* L., var. *altilis* L.) the thermo-electric method was used since it has several distinct advantages. Fresh, untreated tissue may be examined, smaller portions may be used and several samples may be tested at the same time. Thus the material undergoes the minimum amount of physical and chemical changes, is treated more uniformly, and the relationships between smaller regions can be studied.

The results obtained indicate that in general greater values for the freezing point depressions are found in the region one to five centimeters from the tip than in the regions just below. The range of values tended to be high at the tip, decreasing to a point near the soil line where there was a slight rise, which was followed first by a decrease and then by an increase near the crown.

### APPARATUS

The apparatus was essentially the same as that used by Carrick (2). The single junction, copper-constantan thermo-couples were constructed of No. 36 B. & S. gauge wires, wound with silk and coated with a bakelite mixture. The wires were further insulated except for a few centimeters from the junctions, with "spaghetti" as used to cover radio busbar. The working junction and the reference junction were made by soldering the two wires together. (The most convenient procedure was to twist the uncovered tips of the wires tightly together several turns, then cut off all but the last twist, which was cleaned with acid and plunged in melted solder.) The junctions were coated with bakelite. The reference junctions were placed in thin-walled glass tubes several millimeters in diameter and 22 centimeters long, sealed at one end and filled with paraffin. These were kept in a large-mouthed thermos bottle containing finely crushed ice and distilled water. A thick plug of dry absorbent cotton proved a very satisfactory method for closing the opening. On the working junction, the bakelite coating was a little thicker, yet it was not bulky or easily frangible and could very easily be renewed when necessary. The differences in potential between the two junctions were measured by a Leeds and Northrup Type K potentiometer. With this was used a Leeds and Northrup Type R reflec-



ting galvanometer, with a curved scale and telescope which was mounted on the wall. The galvanometer had a sensitivity of 15 microvolts per millimeter at a distance of 1 meter. The source of the working current, a radio battery of 3 cells connected in parallel, maintained a very steady current. By means of a special switch it could be connected either with the potentiometer or with a battery charger. To balance the current Weston Standard cells were used. Although as many as 24 thermo-couples, connected to a bank of 12 two-polar, double-throw switches, could be in operation during a given test, in general only about ten to twenty readings were made at one time.

*Calibration.* To calibrate the apparatus the melting point of 35 cc. of freshly redistilled aniline ( $C_6H_5NH_2$ ) was determined with a number of thermo-couples selected at random. An average E.M.F. of 243.4 microvolts was obtained. If the thermo-electric power of the copper-constantan couple is taken as 39 microvolts per degree Centigrade, the E.M.F. of 243.4 microvolts corresponds to a melting point of  $-6.24^\circ C$ . The value given in the International Critical Tables (1:199) for the melting point of aniline having a boiling point of  $184.4^\circ C$ . is  $-6.2^\circ C$ .

Furthermore, to ascertain the variations of the readings of different thermo-couples, several were attached to a small glass rod in such a way that the junctions, although close together, did not touch and allowed a free circulation of the liquid around them. They were placed in 35 cc. of the solution in a pyrex glass tube, having an internal diameter of 3.0 cm. and a length of approximately 15.0 cm. The tube was plugged with dry absorbent cotton and set in another tube large enough to provide an air space of at least 0.5 cm. This air chamber was surrounded by an ice-salt mixture so that a temperature of  $-4.0^\circ$  to  $-6.0^\circ C$ . was maintained. Readings were made of solutions of glucose ( $C_6H_{12}O_6$ ) and potassium chloride (KCl) having such concentrations as would give freezing point depressions within the range for plant tissue. (This range had been found by preliminary tests made with a Beckmann thermometer using minced tissue, as described by McCool and Millar (10).) The liquid was kept in motion by a stirrer, made of a glass rod bent into a circular loop at the lower end, and propelled by a small motor in an up-and-down direction through such a distance and at such a speed (about once per second) as to maintain the contents of the tube at a uniform temperature and to produce a minimum of heat by friction. Freezing was induced by changing the rate of movement of the stirrer, or by scratching the side of the vessel, or by inoculating with a small ice crystal. The results with the different solutions gave assurance that the readings of the different thermo-couples were uniform. For example, readings in microvolts (1 microvolt being equivalent to  $0.026^\circ C$ .) of three thermo-couples tested four times with different potassium chloride solutions were identical for each test; while in two other tests the reading for

one thermo-couple in each case was one microvolt less than those of the other two thermo-couples.

#### MATERIAL

After preliminary tests with various species of plants, young asparagus shoots were selected. They seemed to be well adapted for this study, since the different regions are large enough to obtain samples of a sufficient size, and the character of the tissue into which the thermo-couple is inserted is very uniform in that the fibro-vascular bundles are scattered through the pith.

The material was obtained through the courtesy of Mr. Samuel Untermeyer from his estate in Yonkers, New York. The shoots were from plants of the Washington variety type grown at least five years in richly fertilized soil on the terraces of a western slope. The humidity of the air above the soil was very high at night and frequently during the day. The rows were ridged high with earth so as to exclude light from the shoots.

The collections were usually made in the early morning and the material was covered loosely with waxed paper and stored in a large metal collecting can until used. The determinations were ordinarily started within two hours, never later than six hours, after collections. That several hours of storage at 18° to 22° C. did not seem to affect appreciably the general character of the results will be shown later.

#### METHOD

That slight differences in the rate of freezing and of thawing, or in the temperatures while frozen, may produce very different physico-chemical changes in plant and animal cells and tissues is mentioned by Stiles (14) in his summary of investigations of the effects of cold temperatures. If the tissue is frozen before extraction the factors mentioned above would influence the results. The amount of pressure applied in the extraction may affect the freezing point of the sap of plant tissue (9). The time at which determinations upon the zones from the same shoot are made is of considerable importance. Any delay in use of samples may cause various changes which will be expressed in the values of the freezing point depressions.

These possible changes are avoided by the thermo-electric method as used in this study. The freezing point determinations of 20 to 24 samples of one or more plants can be accurately made during a short period, in which the samples are subjected to almost identical temperatures over the same time interval. Since the thermo-couples are inserted directly into the tissue, extraction of the sap is not necessary and, since relatively small pieces of tissue are used in this method, the determinations can be made at much closer intervals.

The size of the piece was determined by preliminary tests of potato

tuber. The tissue selected was from the medullary portion which is very uniform and can be obtained in abundance. Cylindrical pieces of different dimensions were cut with cork borers. When ten or more determinations were to be made during the same time interval samples were used having a diameter of at least 1.0 cm. and a length of at least 2.0 cm. For fewer determinations those having a diameter of 0.8 cm. and a length of 1.8 cm. gave satisfactory results. Pieces of smaller mass were not used in this work because the rate of the temperature changes and of crystallization were so great that accurate determinations of the freezing point could be made for only one or two samples during a given test period (6). It is possible to reduce the mass to such an extent that there is no perceptible lag when crystallization occurs.

The temperature of the freezing chamber was maintained by an ice-salt mixture between  $-5^{\circ}$  and  $-8^{\circ}$  C. The temperature most commonly used was  $-6^{\circ}$  C.

The observed freezing points were corrected for the amount of undercooling by the following formula suggested by Harris and Gortner (7) in which  $\Delta'$  is the observed maximum freezing point, 0.0125 the heat of liquefaction of ice and  $u$ , the amount of undercooling.

$$\Delta = \Delta' - 0.0125u\Delta'.$$

*Precautions.* In order to be certain that the value obtained was that of the tissue itself rather than of the surrounding medium, the thermo-couples were inserted one centimeter into the tissue. They usually remained firmly fixed during the entire period of the observations, which was from one-half to two hours, never more than three hours.

The air in the freezing chamber was moist but in order to maintain it at a uniform temperature it was circulated by means of a small fan. This movement of air caused some evaporation from the surfaces of the tissue. To avoid this the sample was dipped in paraffin having a low melting point ( $38^{\circ}$ – $40^{\circ}$  C.), then was wound firmly, but not tightly, with soft, white knitting cotton and dipped again into the paraffin. The sample was pierced to the required depth with a clean fine wire. The thermo-couple was then inserted and was attached more securely to the sample by covering the junction and the sample with coarse net which was fastened to the wire by means of a rubber band.

#### DEPRESSION OF THE FREEZING POINT

Measurements of the freezing point depression of five actively growing asparagus shoots whose tips were entirely below the surface of the soil are given in Table I. For the three shoots *A*, *D*, and *E*, the values at one centimeter and at three centimeters from the tip (portions *a* and *b*) were greater than in regions directly below. There was a fairly regular decrease for

TABLE I  
FREEZING POINT DEPRESSIONS OF ACTIVELY GROWING ASPARAGUS SHOOTS WITH TIPS ENTIRELY BELOW THE SURFACE OF THE SOIL

Time of collection, 1931		May 27 7:15 A.M.				May 27 7:15 A.M.		May 28 7:15 A.M.		May 28 7:15 A.M.		June 9 11:45 A.M.	
Specimen		A		B, C, D, E		B		C		D		E	
Portion of shoot		Thermo-couple insertion, cm. from tip		Δ° C.		Thermo-couple insertion, cm. from tip		Δ° C.		Δ° C.		Δ° C.	
a	0.8	1.17†		1.0†		1.26†		0.94†		1.24†		1.45†	
b	2.8	1.11		3.0		1.17		0.96		1.21		1.32*	
c	4.6	0.94		5.0		1.18		0.98		0.97		1.17*	
d	6.4	0.90		7.0		0.94		1.01		0.91		1.26*	
e	8.2			9.0		0.89		0.97		0.86		0.94*	
f	10.0	0.81		11.0		0.80		0.88		0.92		0.96*	
g	11.8	0.82		13.0		0.79		0.87		1.65		0.94*	
h	13.6	0.84		15.0		0.76		0.96		1.70		0.89*	
i	15.4	1.02		17.0		0.80						0.90	
j	17.2	1.01		19.0		0.88						0.93	
k				21.0		0.83						0.94	
l				23.0		0.94							

\* Portions split longitudinally; average of the values of the two halves  
† Portion of shoot nearest shoot base

\* Portions split longitudinally; average of the values of the two halves.  
† Portion of shoot nearest the surface of soil.

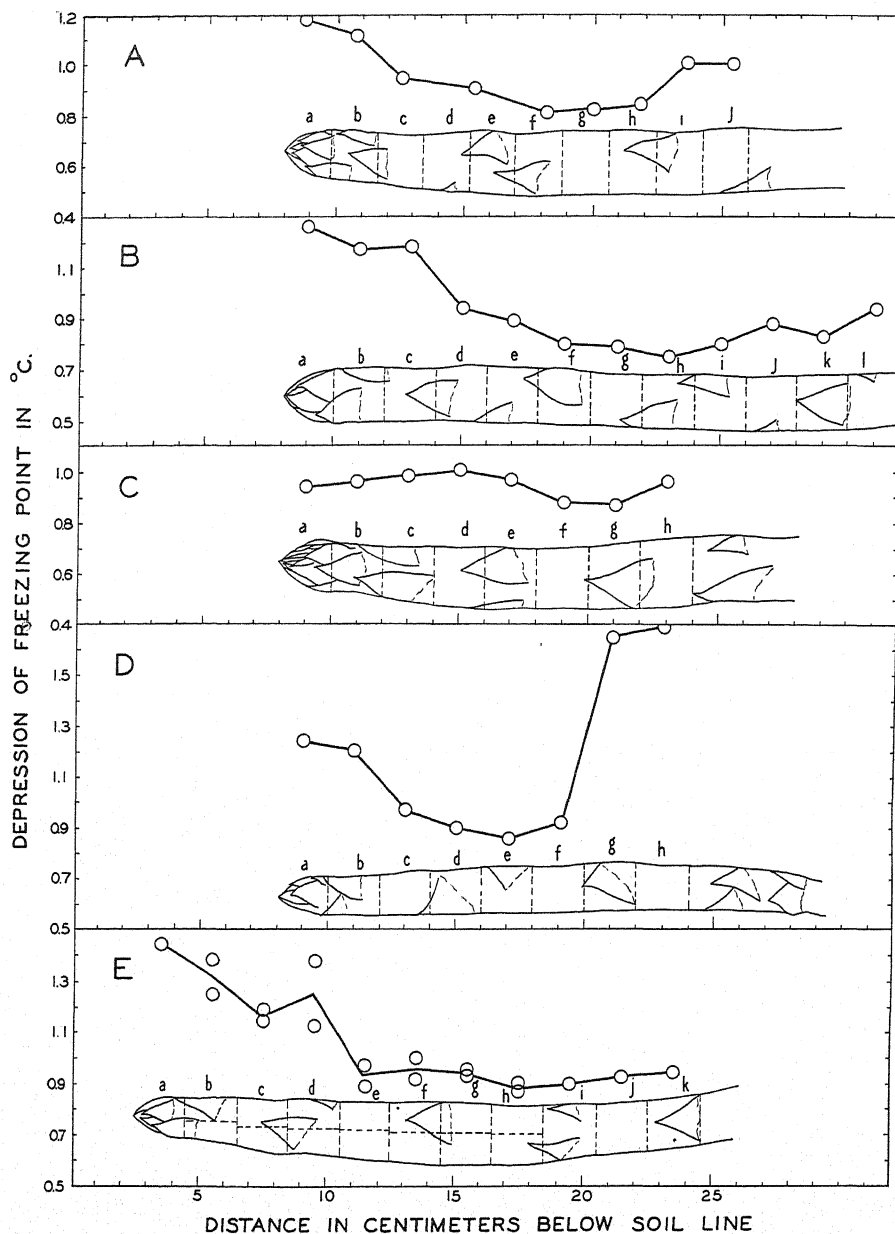


FIGURE 1. The relationship between the values of the freezing point depressions determined at regular intervals from the tip of five asparagus shoots. The tips were entirely below the surface of the soil.

TABLE II  
FREEZING POINT DEPRESSIONS OF ACTIVELY GROWING ASPARAGUS SHOOTS WITH TIPS 1 TO 4 CENTIMETERS ABOVE THE SURFACE OF THE SOIL

Time of collection, 1931	May 14 8:00 A.M.	May 19 7:30 A.M.	May 13 7:45 A.M.	May 26 7:00 A.M.	May 25 7:00 A.M.	May 22 7:00 A.M.	May 25 7:00 A.M.	June 6 7:20 A.M.
Specimen	A	B	C	D	E	F	G	H
Length of shoot above surface of soil	1.5 cm.	1.8 cm.	2.4 cm.	2.5 cm.	2.8 cm.	3.3 cm.	3.4 cm.	3.7 cm.
Portion of shoot section, cm. from tip	Thermo- couple in-	Thermo- couple in-	Thermo- couple in-	Thermo- couple in-	Thermo- couple in-	Thermo- couple in-	Thermo- couple in-	Thermo- couple in-
a	1.0	1.26†	1.11†	1.16	1.37	1.16	1.23	1.02
b	3.0	1.16	1.27	1.44†	1.25†	1.21*†	1.03	0.90*†
c	5.0	0.94	1.16		1.11	1.06*	0.88	0.98*
d	7.0	0.92*		1.14	1.12	0.86	0.84*	0.84*
e	9.0	0.88	1.17	1.26	1.16	0.85	0.95	0.85*
f	11.0	0.98*		0.97		0.93	0.75	0.78*
g	13.0	0.86		1.06		0.75	1.23	
h	15.0		1.00	0.99		0.77		
i	17.0			1.01		0.81		
j	19.0			1.04		0.98		
k	20.0			0.92				
	Δ° C.	Δ° C.	Δ° C.	Δ° C.	Δ° C.	Δ° C.	Δ° C.	Δ° C.

\* Portions split longitudinally; average of the values of the two halves.

† Portion of shoot nearest the surface of the soil.

10 to 15 cm. when the values for regions nearer the crown and roots increased. Shoot *D* increased markedly in this region (portions *g* and *h*). Shoots *A* and *B* were similar in size, shape, and appearance, as indicated by the diagrams in Figure 1. They were growing within a few feet of each other with their tips approximately nine centimeters below the surface of the soil and were collected at the same time. Although the determinations for *B* were made several hours later, the freezing points of the corresponding regions of the shoots are similar as shown by the curves in Figure 1. On

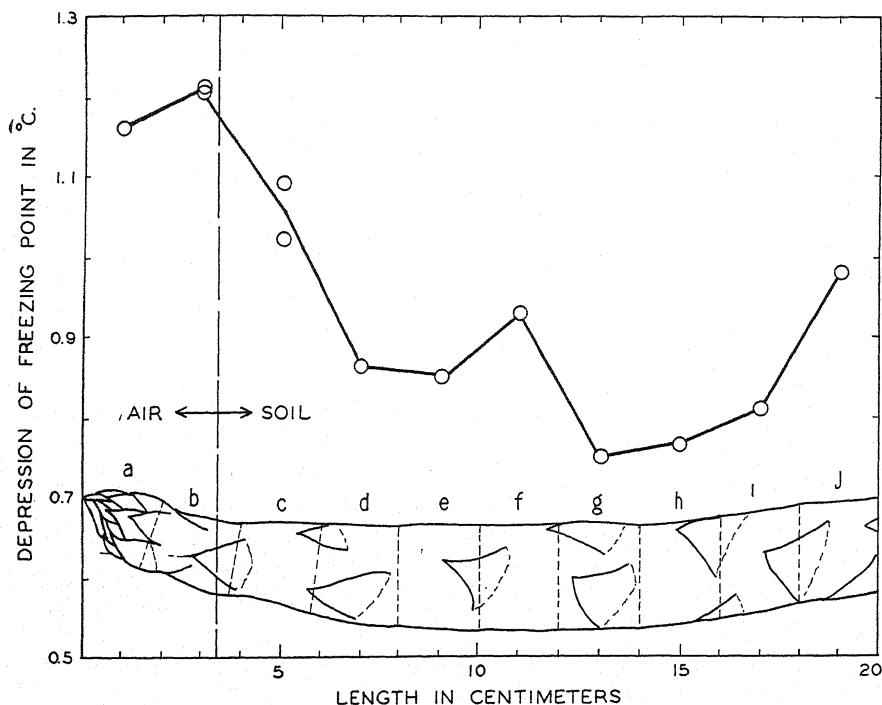


FIGURE 2. The relationship between the values of the freezing point depressions determined at regular intervals from the tip of one asparagus shoot. The tip had emerged 3.3 cm.

the other hand shoots *C* and *D*, which were collected at the same time and had the freezing points determined during the same time interval, differed from each other (curves *C* and *D*, Fig. 1). These shoots were different in appearance. The scales of shoot *D* were smaller, closer together and fitted more tightly over the tip than those of shoot *C*. The values for shoot *E*, which was very similar in appearance to shoots *A*, *B*, and *D*, produced the same general type of curve. These shoots had the aspect of those beginning to elongate, whereas shoot *C* resembled one that had just completed a period of rapid growth. The physiological condition of shoots entirely be-

low the surface of the soil seems to determine to a great extent the distribution of maximum and minimum values. A few additional hours of storage seem to have less effect.

In general a similar distribution of values was observed in shoots the tips of which had emerged from the soil 1.5 to 3.7 cm. (Table II). The greatest value was found in portions one or three centimeters from the tip (portions *a* and *b*). In four shoots, portion *a*, and in three, portion *b*, was the greater. The values for portions near the surface of the soil were relatively greater than for the adjacent portions. The values found for shoot *F* which is typical of the freezing points of these eight shoots are represented graphically in Figure 2.

The maximum and minimum values for shoots whose tips were more than five centimeters above the soil (Table III) were about the same as for shoots just emerging; but their distribution was less regular. In three shoots only (*A*, *F*, and *H*) the greater values were in the portions one and three centimeters from the tip (portions *a* and *b*). The portion nearest the surface of the soil (Table III, symbol †) in six shoots (*A*, *B*, *C*, *D*, *F*, and *H*) had relatively larger values than the adjacent zones.

A greater length of shoot could be examined by making tests of zones three centimeters apart. The freezing points of such zones of two shoots are given in Table IV. The shoots were collected at 11:30 A.M. after three hours of sunshine. The longer shoot (*A*) was succulent for 30 cm.—almost the entire length above the soil. Lateral branches had started to develop in the upper zone down to 15 cm. from the tip. The tip portion (*a*), with the thermo-couples inserted 2 cm. from the tip, had a greater relative depression of the freezing point. There was also a tendency to increase definitely in the portions nearer the soil (Table IV, symbol †). Below the surface of the soil the lower portions had a slight increase in value. The appearance of this shoot together with a graph of the freezing point values is shown in Figure 3. The shorter shoot (*B*) was succulent to 9 cm. from the tip and the zone of lateral branches extended for 8 cm. As in shoot *A* the values for the tip portion (*a*) was higher than for the adjacent portion while at the surface of the soil an increase was present but was not so pronounced. In the shorter shoot more determinations were made of portions below the soil. As found in some of the previously described shoots (Table I, Fig. 1) there was a definite increase in the values near the crown.

#### DISCUSSION

The amount and rate of elongation of one centimeter zones near the upper portion of asparagus shoots have been found by Working (15) in a typical shoot, to be greatest in the second and the third centimeter zones. The fourth centimeter zone elongated somewhat less, and the fifth centimeter zone considerably less. It is interesting that these zones of more



TABLE III  
FREEZING POINT DEPRESSIONS OF ASPARAGUS SHOOTS WITH TIPS 5 TO 21 CENTIMETERS ABOVE THE SURFACE OF THE SOIL

Time of collection, 1931	May 19 7:15 A.M.	May 12 7:15 A.M.	May 9 7:15 A.M.	May 22 7:15 A.M.
Specimen	A	B	C	D
Length of shoot above surface of soil	5.2 cm.	5.3 cm.	6.1 cm.	8.6 cm.
Portion of shoot	Thermo-couple insertion, cm. from tip	Thermo-couple insertion, cm. from tip	Thermo-couple insertion, cm. from tip	Thermo-couple insertion, cm. from tip
	$\Delta^{\circ}\text{C.}$	$\Delta^{\circ}\text{C.}$	$\Delta^{\circ}\text{C.}$	$\Delta^{\circ}\text{C.}$
a	1.0	1.0	1.5	3.0
b	3.0	3.0	3.5	5.0
c	5.0	5.0	5.5	7.0
d	7.0	7.0	7.5	9.0
e	9.0	9.0	9.5	11.0
f	11.0	11.0	11.5	13.0
g		0.96	13.5	15.0
h		1.16	15.5	17.0
i		1.16	17.5	21.0
j		1.16		
k		1.16		

\* Portions split longitudinally; average of the values of the two halves.

† Portion of the shoot nearest the surface of the soil.

TABLE III—Continued

Time of collection, 1931	June 11 11:30 A.M.	May 8 8:45 A.M.	May 7 8:00 A.M.	May 7 8:00 A.M.
Specimen	E	F	G	H
Length of shoot above surface of soil	12.3 cm.	15.6 cm.	19.2 cm.	21.2 cm.
Portion of shoot	Thermo-couple insertion, cm. from tip	Thermo-couple insertion, cm. from tip	Thermo-couple insertion, cm. from tip	Thermo-couple insertion, cm. from tip
	$\Delta^{\circ}\text{C.}$	$\Delta^{\circ}\text{C.}$	$\Delta^{\circ}\text{C.}$	$\Delta^{\circ}\text{C.}$
a	1.0	1.5	1.5	1.5
b	3.0	3.5		
c	5.0	5.5	5.5	5.5
d	7.0	7.5		7.5
e	9.0	9.5		
f	11.0	11.5	11.5	11.5
g	13.0	13.5	13.5	13.5
h	15.0	15.5	15.5	15.5
i	17.0	17.5	17.5	17.5
j	19.0	19.5		19.5
k				21.5
l	21.0	0.90		
m	23.0	0.88		
n	25.0	0.82		
	27.0	0.93		
		0.97	0.95	0.90
		1.14	0.92	1.10
		1.44	0.87	
		0.88	0.84	
		0.77	1.00	
		0.78	1.00	
		0.91†	0.67	
		0.75	0.82†	
		0.88	0.77	
		0.78	0.72	
				0.94
				0.91
				0.79
				0.72
				0.72
				0.67
				0.71
				0.71
				0.71
				0.71
				0.71
				0.77†
				0.77†

TABLE IV  
FREEZING POINT DEPRESSIONS OF TWO ASPARAGUS SHOOTS WITH LATERAL BRANCHES BEGINNING TO DEVELOP

Time of collection		June 12, 1931, 11:30 A.M.			June 13, 1931, 11:30 A.M.		
Specimen		A			B		
Portion of shoot	Thermo-couple insertion, cm. from tip	Thermo-couple insertion, cm. from surface of soil	Condition of portion	$\Delta^{\circ}\text{C.}$	Thermo-couple insertion, cm. from surface of soil	Condition of portion	$\Delta^{\circ}\text{C.}$
a	2.0	+45.5	Succulent	1.12	+8.6	Succulent	1.23
b	4.0	+43.5	"	0.89	+6.6	"	1.04
c	7.0	+40.5	"	0.96	+0.6	"	0.93†
d	10.0	+37.5	"	0.94	-2.4	Slightly tough	1.12*
e	13.0	+34.5	"	0.89	-5.4	Tough	1.25*
f	16.0	+31.5	"	0.84	-8.4	"	0.95*
g	19.0	+28.5	"	0.76	-11.4	"	1.13*
h	22.0	+25.5	"	0.69	-14.4	"	0.98
i	25.0	+22.5	"	0.69	-17.4	"	0.95*
j	28.0	+19.5	"	0.73	-20.4	"	0.98*
k	31.0	+16.5	Slightly tough	0.61	-23.4	Very tough	1.24
l	34.0	+13.5	Tough	0.66			
m	37.0	+10.5	"	0.75			
n	40.0	+7.5	"	0.91			
o	43.0	+4.5	"	1.01			
p	46.0	+1.5	"	1.15			
q	49.0	-0.5	Very tough	1.33†			
r	52.0	-3.5	"	0.86			
s	55.0	-6.5	"	1.06			
Condition of shoot		For distance of 15 cm. from tip lateral branches 1 to 2 cm. in length at all nodes.			Lateral branches 0.5 to 1 cm. for 8 cm. from tip at all nodes.		

\* Portions split longitudinally; average of the values of the two halves.

† Portion of shoot nearest the surface of the soil.

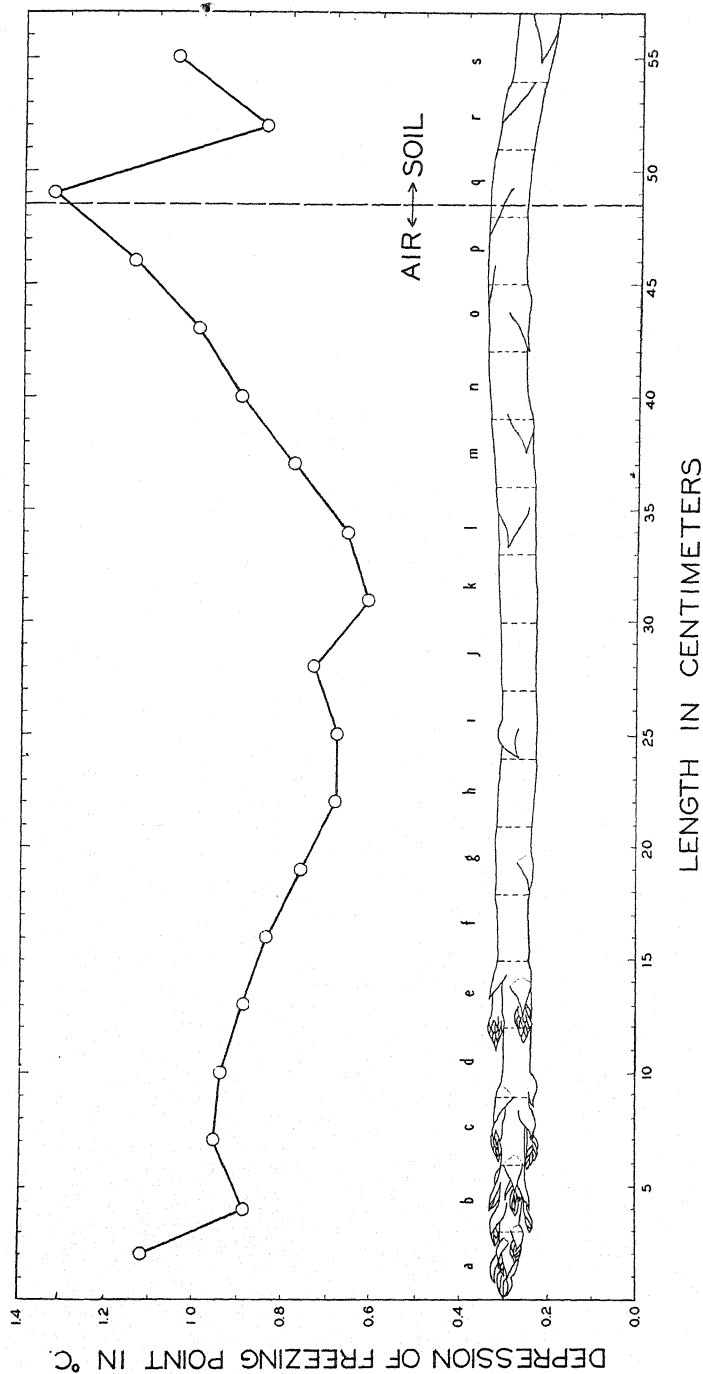


FIGURE 3. The relationship between the values of the freezing point depressions determined at different intervals of an asparagus shoot the tip of which was 42.5 cm. above the surface of the soil.

rapid elongation correspond to the zones in which the greater freezing point depressions are found most frequently in this study.

The terms, freezing point depressions and osmotic pressures, will be used synonymously in this discussion since it is well known that a relationship exists between their values for dilute solutions and for plant tissues. Several observers using different methods have found higher osmotic pressures in portions nearer the apex than in lower portions. Cavara (3), using the Beckmann method, records that a scape of *Agave americana*, 190 centimeters in length, had higher values for the freezing points in the upper 20 centimeters, followed by a decrease in values for the next 30 centimeters when there was an increase again to a high value at the base. He obtained similar results with a 170 centimeter scape of the same species of *Agave* and a 19 centimeter stem of *Opuntia ficus-indica*. Many of his determinations were made by inserting the thermometer directly into the tissue. It is interesting that the curve of values for the asparagus shoot shown in Figure 3 almost parallels his curves. The works of others who have more recently observed similar high values in regions near the tip have been reviewed and discussed in an earlier paper (5).

These greater osmotic values are probably due to the presence of soluble substances that can produce growth. Eckerson (4) has found that throughout the growth of the wheat plant "the young parenchyma cells (the meristem) of any growing region contain a greater amount of fructose and of asparagine than the adjacent parenchyma cells." That larger amounts of simple carbohydrates and of the less complex forms of organic nitrogen compounds are present in the central meristematic portion of the paper-white narcissus bulb than in the storage tissue of the scales is stated by Nightingale and Robbins (12).

The fact that greater values are found nearer the crown and roots is easily explained. Roots and rhizomes of asparagus plants of various ages and at different periods of the year may have from 18 to 40 per cent of sugars in the dry matter. Also there is a definite decrease in the soluble carbohydrates in these regions after the cutting season indicating that there has been a movement of these substances into the shoots (11, 13).

The differences ranged as high as  $0.70^{\circ}$  C., and in the majority of cases were more than  $0.20^{\circ}$  C. between the high, low, and intermediate values for freezing point depressions. The average difference between duplicate determinations of 27 different zones was  $0.07^{\circ}$  C. (The samples for these duplicate determinations were obtained by splitting a two centimeter portion longitudinally into halves. In the tables the values for the averages of two such determinations are marked with the symbol\*.)

At present no satisfactory reason can be given to account for the appearance of the increase in values near the surface of the soil. That the upper seven inches of the soil changes in temperature much more rapidly

than the soil below and less rapidly than the air above has been found by Bouyoucos (1) after making continuous records of soil temperatures over long periods of time. That the moisture and temperature conditions under which the plants have been grown may have greater effect is emphasized by Jones and Robbins (8). Therefore these external factors in addition to the internal conditions should be considered.

All the chemical analyses recorded for this plant have been made of large portions and composite samples of several plants. It will be necessary to study either composite samples of small zones from many plants by macrochemical methods or single small zones by microchemical methods before one can make any definite conclusions regarding the cause for the differences found in osmotic pressure. Analyses for soluble carbohydrates and nitrogen compounds should give interesting correlations. In such studies, not only the shoots of different lengths but the rhizomes and roots of the same plants should be carefully examined.

#### SUMMARY

1. Freezing point depressions of actively growing shoots of asparagus were determined by a thermo-electric method. Copper-constantan thermocouples were inserted one centimeter into portions cut from the asparagus shoots. The portions were usually 2 cm. in length and 1 cm. in diameter.
2. The values for the freezing point depressions tended to be highest in regions 1 cm. and 3 cm. from the tip of asparagus shoots still below the surface of the soil.
3. A similar distribution of values was observed in the majority of shoots whose tips had emerged from the soil 1.5 to 3.7 cm.
4. The distribution of the maximum and minimum values was less regular in shoots whose tips were more than 5 cm. above the soil.
5. In all types of shoots there was a tendency for the values to increase near the crown and roots. Also there was slight increase near the surface of the soil.

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# THE EFFECT OF VARIOUS CHEMICAL TREATMENTS OF DORMANT POTATO TUBERS ON THE PEROXIDASE, CATALASE, pH, AND REDUCING PROPERTIES OF THE EXPRESSED JUICE<sup>1</sup>

JOHN D. GUTHRIE

It has been shown by Denny (2, 3) that ethylene chlorhydrin, thiourea, and sodium thiocyanate are very effective in breaking the dormancy of potato tubers. It has also been found (4) that these chemicals produce changes in peroxidase, catalase, pH, and reducing properties of the expressed juice. It was shown that the correlation was not good between the effect of the chemical treatments on catalase, pH, and reducing properties, and the effect of the treatments on dormancy. For example, ethylene chlorhydrin produced a marked change in pH and in reducing properties, while thiourea and sodium thiocyanate, although very effective in breaking dormancy, produced only very small changes in pH and in reducing properties. Ethylene chlorhydrin produced an increase in catalase, thiocyanate an increase in catalase if the juice was dialyzed, while thiourea produced little or no change in catalase. However, all three chemicals increased peroxidase.

The present paper reports the results of extending this work to include a number of other chemicals, some of which are not very effective in breaking dormancy, others which are effective, but less so than the chemicals previously studied. Especial interest was centered in peroxidase, since the three chemicals previously studied increased it. It was also hoped that some clue might be found as to the relationship between the nature of the compound used and its power to break dormancy.

## CHEMICALS INVESTIGATED

In general, a chemical to be used effectively in the treatment of potatoes must either be soluble in water, so that a soak treatment may be used, or be sufficiently volatile for a vapor treatment to be applied. If both soluble in water and volatile a dip treatment may be used. The reasons for selecting the particular chemicals used are as follows:

Ethyl alcohol ( $\text{CH}_3\text{CH}_2\text{OH}$ ). Jesenko (8) found ethyl alcohol effective in breaking dormancy of woody plants. It is related chemically to ethylene chlorhydrin.

Acetaldehyde ( $\text{CH}_3\text{CHO}$ ). This compound was found effective in breaking the dormancy of woody plants by Boresch (1). It was desired to include a chemical having an aldehyde group.

<sup>1</sup>Herman Frasch Foundation for Research in Agricultural Chemistry, Paper No. 27.



Hydrogen cyanide (HCN). Gassner (5) found hydrogen cyanide effective in breaking dormancy of woody plants. Its effect on respiration and on oxidative processes in general has received a large amount of study.

Methyl thiocyanate ( $\text{CH}_3\text{SCN}$ ). This compound was tried because of its obvious relationship to potassium thiocyanate.

Acrolein ( $\text{CH}_2\text{:CH.CHO}$ ). It was thought desirable to include an unsaturated compound. Since such compounds are often very toxic to plants, it was expected that low concentrations might be effective.

Potassium cyanide (KCN). This was tried for the same reasons as hydrogen cyanide.

Potassium cyanate (KOCN). This is the oxygen analog of potassium thiocyanate.

Urea ( $\text{NH}_2)_2\text{CO}$ ). This is the oxygen analog of thiourea.

Sodium chloracetate ( $\text{CH}_2\text{Cl.COONa}$ ). Chloracetic acid is related chemically to ethylene chlorhydrin.

Ethylene cyanhydrin ( $\text{CH}_2\text{CN.CH}_2\text{OH}$ ). This was used because of its similarity to ethylene chlorhydrin.

#### METHODS

Treatments were applied according to the soak, dip, and vapor methods of Denny (3). Potatoes (*Solanum tuberosum* L.) were treated in series of five lots each. One lot was the check, another a treatment with ethylene chlorhydrin, potassium thiocyanate, or thiourea, and the other three treatments with different concentrations of the chemical under investigation. The varieties Irish Cobbler and Bliss Triumph were used. There is no essential difference in the response of these varieties to chemical treatments, either in the chemical changes observed or in the breaking of dormancy. After treatment the pieces were planted and samples taken for analysis after four to six days. Twelve pieces were planted in order to see what effect the treatment had on growth. After about three weeks the pieces were dug up and sprout length measured. They were then replanted and photographs taken after the plants had grown. In this way the chemical treatments could be classified according to their effectiveness in breaking dormancy.

The pieces taken for analyses were peeled, ground through the nut cutter of a food chopper, the juice squeezed through cheesecloth, poured at once into centrifuge tubes and centrifuged. Peroxidase, catalase, pH, reduction of methylene blue, and reduction of iodine in acid solution were determined. The methods of analyses have been described in previous papers (4, 6).

#### RESULTS

Since three or more series of treatments of five lots each were made for each chemical studied, the data will not be published in detail. Table I,

TABLE I  
THE EFFECT OF ETHYL ALCOHOL ON DORMANT IRISH COBBLER POTATOES

24 hr. dip treatments	5 days after treatment					Sprouting data 15 days after treatment	
	pH	N/100 I cc.	Reduction of methylene blue	Peroxidase	Catalase	No. sprouts per 12 pieces	Total length cm.
Ethylene chlorhydrin 30 cc./l.*	6.86	5.6	++++	5.40	28.0	12	37
Ethyl alcohol 80 cc./l.	6.42	2.2	+++	2.13	17.5	12	7
Ethyl alcohol 40 cc./l.	6.31	1.8	+++	2.36	14.5	12	3
Ethyl alcohol 20 cc./l.	6.24	1.4	++	1.94	11.9	7	1
Check—H <sub>2</sub> O	6.07	0.8	—	2.18	10.0	2	0.2

\* 40 per cent solution.

TABLE II  
THE EFFECT OF ACETALDEHYDE TREATMENT ON DORMANT BLISS TRIUMPH POTATOES

20 hr. vapor treatments	5 days after treatment					Sprouting data 24 days after treatment	
	pH	N/100 I cc.	Reduction of methylene blue	Peroxidase	Catalase	No. sprouts per 12 pieces	Total length cm.
Ethylene chlorhydrin 1 cc./l.*	6.90	6.5	++++	10.70	17.3	9	31
Acetaldehyde 4/5 cc./l.	6.36	2.1	+++	1.99	6.5	Rotted	—
Acetaldehyde 2/5 cc./l.	6.31	1.6	++	2.04	6.2	7	11
Acetaldehyde 1/5 cc./l.	6.27	1.2	±	1.87	4.6	11	3
Check—closed container	6.12	0.6	—	1.85	3.6	1	0.1

\* 40 per cent solution.

showing the effect of ethyl alcohol dip treatments, and Table II, showing the effect of acetaldehyde vapor treatments, are examples of how the data were tabulated. It will be noted that both ethyl alcohol and acetaldehyde produce marked changes in pH and in reducing properties of the juice. In this respect they act similarly to ethylene chlorhydrin, although the changes produced are not so large. The effect of the treatments recorded in Tables I and II on growth is shown in Figure 1.

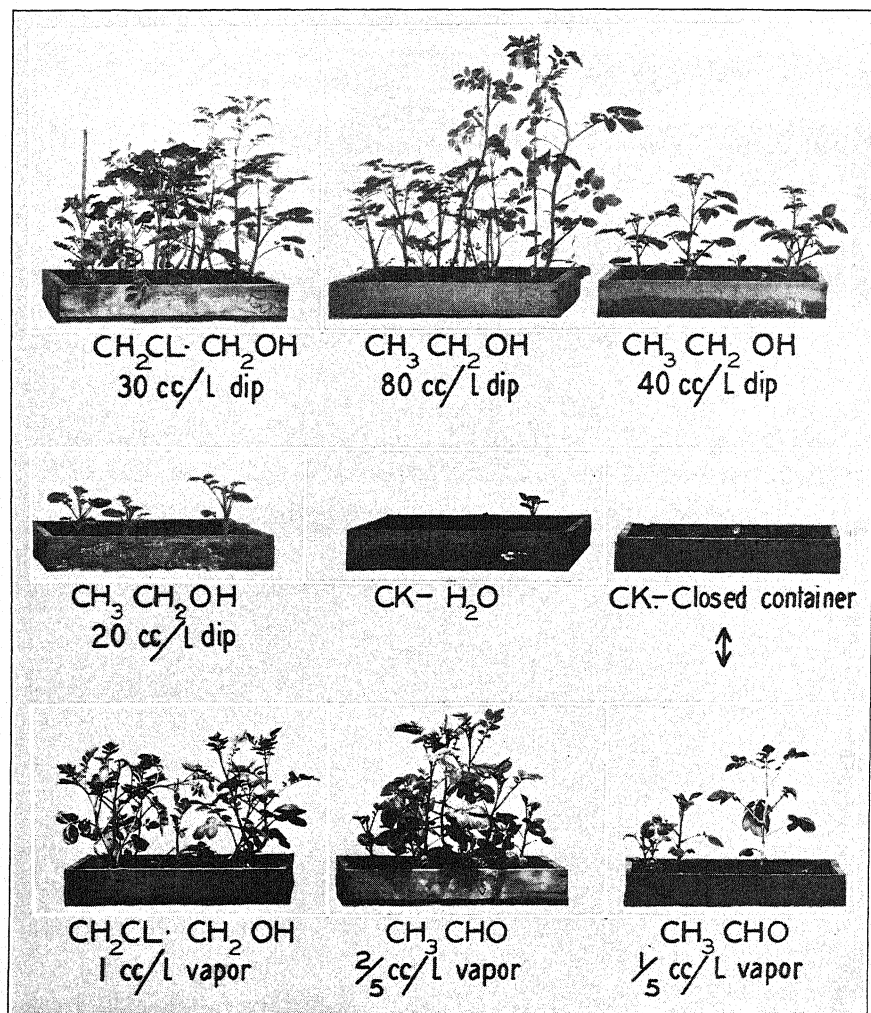


FIGURE 1. The effect of ethylene chlorhydrin ( $\text{CH}_2\text{ClCH}_2\text{OH}$ ), ethyl alcohol ( $\text{CH}_3\text{CH}_2\text{OH}$ ), and acetaldehyde ( $\text{CH}_3\text{CHO}$ ) on the growth of dormant potatoes.

TABLE III  
THE EFFECT OF VARIOUS CHEMICAL TREATMENTS ON THE PROPERTIES OF THE EXPRESSED JUICE AND ON GROWTH  
OF DORMANT POTATO TUBERS

Chemical	Formula	Method of treatment	Favorable concentration per l.	Change in					Catalase	Growth
				pH	N/100 I cc.	Reduction of methylene blue	Peroxi-dase			
Ethylene chlorhydrin	$\text{CH}_2\text{Cl} \cdot \text{CH}_2\text{OH}$	24 hr. dip	30 cc.**	0.73	4.3	+	3.02	16.3	+	+
Ethylene chlorhydrin	$\text{CH}_2\text{Cl} \cdot \text{CH}_2\text{OH}$	24 hr. vapor	1 cc.**	0.53	3.0	+	4.25	14.1	+	+
Potassium thiocyanate	KSCN	1 hr. soak	10 g.	0.08	0.2	+	1.55	*	+	+
Thiourea	$(\text{NH}_2)_2\text{CS}$	1 hr. soak	10 g.	0.06	*	±	1.17	2.1	+	+
Ethyl alcohol	$\text{CH}_3\text{CH}_2\text{OH}$	24 hr. dip	80 cc.	0.31	1.1	+	0.00	5.0	+	+
Ethyl alcohol	$\text{CH}_3\text{CH}_2\text{OH}$	24 hr. vapor	3 cc.	0.27	1.4	+	-0.18	5.4	+	+
Acetaldehyde	$\text{CH}_3\text{CHO}$	24 hr. vapor	0.5 cc.	0.36	1.1	+	0.57	6.7	+	+
Ethylene chlorhydrin	$\text{CH}_2\text{Cl} \cdot \text{CH}_2\text{OH}$	1 hr. soak	3 cc.**	0.28	1.0	+	1.07	7.8	+	+
Hydrocyanic acid	HCN	24 hr. vapor	0.1 g.	0.20	0.4	±	1.12	6.4	+	+
Acetaldehyde	$\text{CH}_3\text{CHO}$	24 hr. dip	20 cc.	0.15	0.5	±	0.30	0.0	+	+
Methyl thiocyanate	$\text{CH}_3\text{SCN}$	24 hr. vapor	0.1 cc.	0.21	0.3	+	0.90	6.0	+	+
Ethyl alcohol	$\text{CH}_3\text{CH}_2\text{OH}$	1 hr. soak	20 cc.	0.09	0.6	±	-0.67	2.7	+	+
Acrolein	$\text{CH}_2=\text{CH} \cdot \text{CHO}$	24 hr. vapor	.01 cc.	0.05	0.1	-	0.29	0.7	+	+
Potassium cyanide	KCN	1 hr. soak	3 g.	0.16	0.0	-	0.50	6.7	±	±
Potassium chloride	KCl	1 hr. soak	40 g.	0.08	0.0	-	1.80	2.9	±	±
Potassium cyanate	KCNO	1 hr. soak	10 g.	0.08	0.2	-	2.33	3.6	±	±
Urea	$(\text{NH}_2)_2\text{CO}$	1 hr. soak	40 g.	-0.09	0.0	-	0.15	0.0	±	±
Sodium chloracetate	$\text{CH}_2\text{Cl} \cdot \text{COONa}$	1 hr. soak	1 g.	0.00	0.0	-	0.66	1.1	±	±
Ethylene cyanhydrin	$\text{CH}_2\text{CN} \cdot \text{CH}_2\text{OH}$	1 hr. soak	10 g.	0.03	0.3	-	0.54	5.6	±	±

\* Chemical absorbed during treatment interferes with detection.

\* Chemical absorbed during treatment interferes with determination.  
\*\* 40 per cent solution.

In order to summarize the results obtained with the various chemicals studied, the differences between the most favorable treatment and the check were calculated. The averages of these changes were found and tabulated in Table III. From an examination of this table a correlation is evident between the pH change and the change in reducing properties. Thus ethylene chlorhydrin, ethyl alcohol, and acetaldehyde increase the pH, the power to react with iodine in acid solution, and the power to reduce methylene blue. This correlation is shown graphically in Figure 2, where the changes in pH are plotted against the change in the power of the juice to

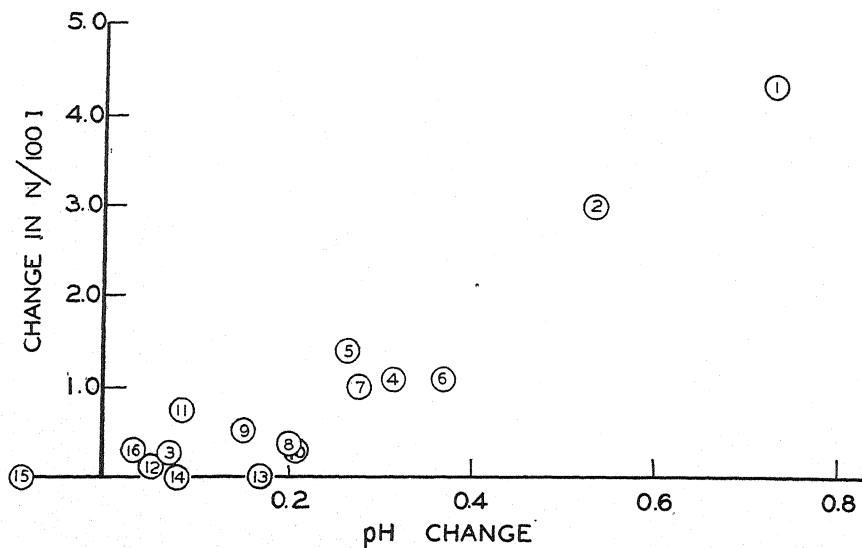


FIGURE 2. The correlation between the pH change and the change in N/100 I titration after treatments: (1) Ethylene chlorhydrin dip, (2) ethylene chlorhydrin vapor, (3) potassium thiocyanate soak, (4) ethyl alcohol dip, (5) ethyl alcohol vapor, (6) acetaldehyde vapor, (7) ethylene chlorhydrin soak, (8) hydrocyanic acid vapor, (9) acetaldehyde dip, (10) methyl thiocyanate vapor, (11) ethyl alcohol soak, (12) acrolein vapor, (13) potassium cyanide soak, (14) potassium chloride soak, (15) urea soak, (16) ethylene cyanhydrin soak.

react with iodine in acid solution. The data are from Table III. It will be seen that those chemicals which produce marked changes in pH, are also very effective in increasing the power of the juice to reduce iodine.

The reason for this correlation is brought out by an experiment summarized in Table IV. Juice of untreated tubers and tubers treated with ethylene chlorhydrin were brought to different pH values by addition of dilute NaOH or HCl, placed in tall containers, and iodine titrations made on aliquots at the start, after 15 minutes and after 45 minutes. It will be seen that while changing the pH of the two juices produces a marked effect on the iodine titration, the treated remains higher than the check. How-

ever, when we consider that the treatments effect the pH change in the tissue, and that the juice only partially represents the tissue, we have good reason to think that change in reducing properties may be a result of the pH change. The converse, that the pH change might be due to the change in reducing properties, has been shown by Miller (10) to be untenable, since the pH difference is present after the difference in reducing properties has been destroyed by aeration of the juice.

TABLE IV  
THE EFFECT OF CHANGING THE pH OF THE JUICE OF UNTREATED AND ETHYLENE CHLORHYDRIN TREATED POTATOES

	pH	N/100 I titration			Nitroprusside test after 45 min.
		At once	15 min.	45 min.	
20 cc. juice of untreated potatoes + 4 cc. N/10 NaOH	7.52	0.6	0.9	1.2	+
20 cc. juice of untreated potatoes + 4 cc. N/20 NaOH	6.76	0.5	0.6	0.8	—
20 cc. juice of untreated potatoes + 4 cc. H <sub>2</sub> O	6.22	0.5	0.5	0.6	—
20 cc. juice of untreated potatoes + 4 cc. N/20 HCl	5.90	0.5	0.5	0.5	—
20 cc. juice of treated potatoes + 2 cc. N/10 NaOH	7.17	1.7	2.8	3.0	+++
20 cc. juice of treated potatoes + 2 cc. H <sub>2</sub> O	6.53	1.6	1.9	2.0	+
20 cc. juice of treated potatoes + 2 cc. N/10 HCl	6.10	1.5	1.4	1.4	+

#### DISCUSSION

An examination of the data presented in Table III reveals no good correlation between the effect of the treatments on growth and the changes in pH, reducing power, peroxidase, or catalase. Although thiocyanate and thiourea produce little change in pH, they are very effective in breaking dormancy. Likewise, thiourea produces little change in catalase. This is in agreement with previous results (4). Ethyl alcohol, which is only slightly less effective in breaking dormancy, produces no increase, perhaps a slight decrease in peroxidase. Potassium chloride, although it is only effective on tubers that are not very dormant, produces an increase in peroxidase.

It will be noted that of the chemicals studied, those most effective in breaking dormancy are either sulphur compounds or produce a distinct change in pH. Thus ethylene chlorhydrin, acetaldehyde, and ethyl alcohol treatments bring about changes in pH of over 0.25 of a pH unit. It has been pointed out that the increase in pH is accompanied by an increase in the power to reduce iodine in acid solution. Thus ethylene chlorhydrin, ethyl alcohol, and acetaldehyde treatments produce increases of more than one cc. in the iodine titration. Tunnicliffe (11) recommends the reduction of iodine in acid solution as a method for estimating sulphhydryl compounds. His method has been widely used for this purpose. Although unpublished evidence indicates that the iodine-reducing substances of the potato are

only partly of a sulphydryl nature, the use of a more specific reaction for sulphydryl compounds, the reduction of sulphur to form hydrogen sulphide, shows that ethylene chlorhydrin treatments increase the sulphydryl content of the expressed juice (7). An increased nitroprusside test may also be observed. This point seems to be of importance in view of the work of Miller (9) who finds a number of sulphur compounds, including sulphydryl compounds, very effective in breaking dormancy.

The chemicals found effective can be conveniently divided into two classes: (1) those that produce an increase in pH and hence an increase in sulphydryl compounds as indicated by an increase in the power of the juice to reduce iodine in acid solution; (2) those that produce only small changes in pH and reducing properties. The first group contains ethylene chlorhydrin, ethyl alcohol, and acetaldehyde, the second such compounds as thiourea and potassium thiocyanate. The problem of how such different chemicals break dormancy is somewhat simplified by such a classification. If we consider the first group as acting by increasing the sulphydryl content of the tubers, the problem of how ethylene chlorhydrin, ethyl alcohol, acetaldehyde, thiourea, and potassium thiocyanate break dormancy resolves itself into a consideration of how RSH, thiourea, and potassium thiocyanate break dormancy.

#### SUMMARY

The effect of a number of chemicals on peroxidase, catalase, pH, and reducing properties of the juice of dormant potatoes has been studied. None of these properties showed good correlation with the breaking of dormancy. The change in pH and the change in reducing properties were found to be correlated. Evidence is given indicating that the increased reducing power may be a result of the change in pH. The chemicals found to be effective up to the present time are either sulphur compounds or compounds which increase the pH and the capacity of the juice to reduce iodine in acid solution. It is pointed out that an increase in sulphydryl compounds is probably involved in this increased iodine titration.

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## THE FUNGICIDAL ACTION OF SULPHUR. III. PHYSICAL FACTORS AFFECTING THE EFFICIENCY OF DUSTS<sup>1</sup>

FRANK WILCOXON AND S. E. A. MCCALLAN

In the previous paper of this series (7) studies were made of the interaction of sulphur with spores from a number of species of pathogenic fungi, in order to throw light on the mechanism by which it acts as a fungicide. The same type of sulphur of U. S. P. grade was used throughout. In the present work the fungicidal effect of a number of different commercial sulphurs has been investigated, using spores of a single fungous species in order to find out whether there exists a significant difference in the efficiency of different commercial preparations, and if so, what causes give rise to this difference.

It was found by means of laboratory toxicity experiments that commercial sulphurs fall into groups which do differ significantly in toxicity, when tested under similar conditions. It was thought at first that such differences might arise from an inherent difference in the rate of formation of hydrogen sulphide when various sulphurs were caused to react with spores of a given species of fungus since previous work (7) indicates that this gas is the actual fungicidal agent.

Experiments on the rate of formation of hydrogen sulphide, using the method described in a previous paper (7, p. 19), showed that the method was not satisfactory when working with sulphurs containing impurities. For example, iron in the sulphur was found to unite with the hydrogen sulphide formed so that the lead acetate paper remained uncolored, and failed to indicate the true rate of hydrogen sulphide evolution.

When using the small glass cells previously illustrated (7, p. 27), no appreciable difference in the rate of hydrogen sulphide evolution with various commercial sulphurs could be detected. The differences in toxicity therefore must be sought in some other property.

### CLASSIFICATION OF SULPHUR FUNGICIDES

The examination of numerous fungicides now on the market, whose principal active constituent is elementary sulphur, showed that they might be classified into two broad groups; the first of these groups comprises preparations intended to be applied as dusts, while those of the second group disperse more or less readily in water, and are intended to be applied as a spray. The members of the first group may be further subdivided into dusts which consist of ground sulphur with no additional substances, except a small amount of unavoidable impurities, and dusts to which vari-

<sup>1</sup> Herman Frasch Foundation for Research in Agricultural Chemistry, Paper No. 26.

ous other materials have been added intentionally to modify the properties of the sulphur. Examples of such substances are gums, proteins, infusorial earth, bentonite, etc. The second group, consisting of sulphurs for use as a spray, may be divided into wettable sulphurs, which disperse in water, but form relatively coarse and unstable suspensions, and the colloidal sulphurs, which contain many particles so small that they remain in suspension for many days.

In this study the samples selected were chosen in such a way that they included representatives of all these classes, as shown in Table I. The sam-

TABLE I  
CLASSIFICATION OF SULPHUR FUNGICIDES WITH EXAMPLES STUDIED

Classification		Brand	Manufacturer
Dusts	Straight	200 Mesh Sulphur	Niagara Sprayer and Chemical Co. Middleport, N. Y.
		Dusting Sulphur	Ansbacher-Siegle Corp. New York, N. Y.
		Aero Smoke	American Cyanamid Co. New York, N. Y.
		300-7 Dusting Sulphur	National Sulphur Co. New York, N. Y.
	Modified	Sulpho-Tone	Lucas Kil-Tone Co. Vineland, N. J.
		Orchard Brand Dritomic Sulphur Kolodust	General Chemical Co. New York, N. Y. Niagara Sprayer and Chemical Co. Middleport, N. Y.
Sprays	Suspensions	Flotation Ferrox Sulphur	Koppers Products Co. Pittsburgh, Pa.
		Mulsoid-Sulphur	Sherwin Williams Co. Cleveland, Ohio
	Colloids	Colloidal Sulphur Ialine	Premier Mill Corp. Geneva, N. Y. Burt, Boulton and Haywood, Ltd. London, England

ples were obtained directly from the manufacturers, and the results refer to the particular sample furnished. No study was made of the uniformity of samples of a given brand at different periods of time.

#### METHODS

The method of determining toxicity by means of the spore germination tests, previously used and discussed in detail (6, 7, 15) has been employed in this study. Here interest is centered largely on an investigation of the distribution of sulphur particles, the spatial relations of particles and spores, and the toxicological evaluation of fungicides differing but little. This requires that a large number of spores and particles distributed more or

less on one plane be counted. For this purpose the moist chamber method is well adapted, whereas the hanging drop method employed by many workers in toxicity studies is entirely unsuited and inadequate.

Conidia of the American brown rot fungus, *Sclerotinia americana* (Wormald) Norton and Ezekiel, have been used throughout. As has been shown in the two previous papers (7, 15) the spores of this fungus are well adapted for laboratory toxicity tests. In addition, for comparative tests it is desirable to have a fungus the spores of which are not unduly sensitive nor resistant to the toxic agent. *Sclerotinia americana* is intermediate in this respect as regards sulphur dust. The conditions for germination were

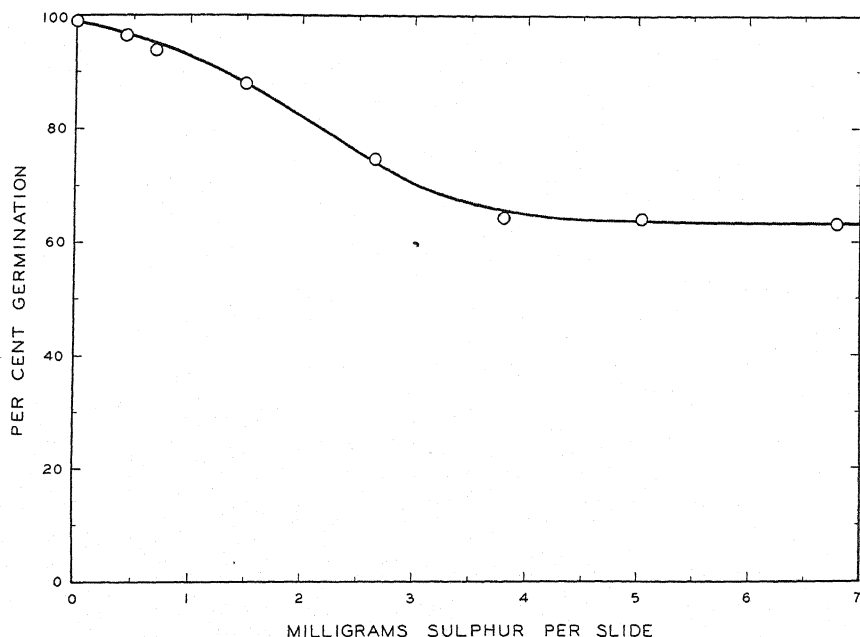


FIGURE 1. A typical sulphur dust toxicity curve. Note the flattening out. Germination of conidia of *Sclerotinia americana*.

essentially the same as those reported in the earlier papers, the spores being from 6 to 7 days old, unless otherwise stated, and from cultures of the same isolation as previously described (7, 15). The temperatures were from 22° to 27° C. and the average variation for any one experiment was less than 2° C.

The sulphur dusts were applied to the glass slides by means of the "test tube duster" (6), care being taken to get a uniform distribution. Limited experiments by McCallan (6) had indicated that beyond a certain minimum application of sulphur, additional amounts do not appre-

ciably increase the fungicidal efficiency. A more detailed study has been made of the relation between toxicity and amount of sulphur applied and the results are shown in the typical sulphur toxicity curve of Figure 1. It is seen that the first portion of the curve resembles that of a typical sigmoid toxicity curve (15), but differs in that the latter portion flattens out. Thus in comparative tests at low concentrations it is essential to have the same quantity of dust. At high concentrations only approximately similar amounts are necessary. In this study the quantity of dust applied to each slide has been determined by direct weighing. Where comparative tests of different sulphurs were made at a given weight eight slides were dusted and about 2400 spores counted for each sample of sulphur. This method permits a highly significant difference where the actual percentage germination difference is relatively small. In the case of the toxicity curves, each point is the result of at least two duplicate slides and is based on counts of from 600 to 1200 spores.

Since the greater part of the practice of plant disease control by spraying and dusting is an effort to protect the as yet healthy plant from possible infection by pathogenes and since most phytopathogenic fungi are disseminated and infect during and immediately following rainy periods, it is evident that the protectant (13) is called upon to exercise its greatest usefulness after having been subjected to rain. Accordingly an artificial rain test has been devised, for any critical comparison of sulphur dusts must attempt to evaluate their adhesiveness and fungicidal efficiency after such an exposure.

Distilled water, contained in a carboy, with a head of about 20 inches, was allowed to flow out through a glass tube pointed upward and drawn out to a fine opening. A set screw regulated the force so that the water was driven up to a height of 10 inches in the form of a spray. The spray or "rain" then fell about 50 inches on to the slides. Each slide was held in turn and subjected to a 30 second "rain". During the first 15 seconds one end of the slide was elevated so that the slide was about 20° off the horizontal, the other end was then elevated for the latter 15 seconds. During this 30 second "rain" about 0.3 inches of "rain" fell. As will be shown later by far the greater part of the dust was removed during this treatment which is probably more severe than a 0.3 inch rain in nature. The slides were allowed to dry and spore germination tests performed as usual.

#### RESULTS OF TOXICITY TESTS BEFORE AND AFTER RAIN

The results of toxicity tests with various straight and modified dusts without being subjected to a "rain" test and after such a test are shown in Tables II and III. A comparison of some sulphur sprays with one of the more toxic straight dusts appears in Table IV. These data have been examined by Fisher's method for the "Comparison of Two Means" (3, p. 107).

Here  $x$  is the per cent germination on one slide, that is, the results of counts on about 300 spores, and  $n$  is 14. In the tables a heavy line has been drawn between adjacent dusts which have been found to differ very significantly, that is, with odds of 100 to 1 or greater.

TABLE II  
THE TOXICITY OF SULPHUR DUSTS, WITHOUT BEING SUBJECTED TO A RAIN TEST, TO THE CONIDIA OF *SCLEROTINIA AMERICANA*

Dust	Per cent germination			
	Exp. 1	Exp. 2	Exp. 3	Exp. 4
Dritomic	9.5	10.2	—	16.7
Kolodust	—	15.1	—	28.0
<hr/> *				
Sulpho-Tone	25.6	34.3	—	48.4
National 300-7	—	—	39.9	—
<hr/>				
Aero Smoke	—	—	46.9	—
Ansbacher-Siegle	—	—	46.9	—
<hr/>				
Niagara 200	39.6	50.5	54.0	74.0
<hr/>				
Control	97.5	97.4	97.9	98.0

\* The heavy line between adjacent dusts indicates a significant difference with odds of 100 to 1, or greater.

In Table II, where the dusts have not been subjected to a rain, it appears that the modified dusts are superior to the straight dusts. However, after the rain test (Table III), which gives a truer evaluation of their relative merits, it is seen that the straight dusts are at least equal in toxicity to the modified dusts. Apparently Kolodust retains its effectiveness after

TABLE III  
THE TOXICITY OF SULPHUR DUSTS, AFTER A RAIN TEST, TO THE CONIDIA OF *SCLEROTINIA AMERICANA*

Dust	Per cent germination		
	Exp. 1	Exp. 2	Exp. 3
National 300-7	—	50.0	82.0
<hr/> *			
Kolodust	27.0	65.2	85.4
Aero Smoke	—	66.5	85.1
Ansbacher-Siegle	—	72.0	87.7
Dritomic	59.4	72.3	89.6
<hr/>			
Sulpho-Tone	68.5	82.8	93.2
Niagara 200	80.9	83.8	94.3
<hr/>			
Control	96.2	97.6	99.1

\* The heavy line between adjacent dusts indicates a significant difference with odds of 100 to 1, or greater.

the rain test better than the other modified dusts. A possible explanation for this lies in the fact that the modification has actually produced a change in the physical state of the sulphur<sup>2</sup> and therefore it has qualities superior to that of sulphur dusts modified merely by mechanical additions. It is to be noted that the order of toxicity for the straight dusts with respect to each other is identical in each table.

TABLE IV

THE COMPARATIVE TOXICITY OF SULPHUR SPRAYS AND A SULPHUR DUST, AFTER A RAIN TEST, TO THE CONIDIA OF *SCLEROTINIA AMERICANA*

Fungicide	Per cent germination	
	Exp. 1	Exp. 2
Ialine*	16.4	11.0
Premier Mill	39.5	44.6
Ferrox	42.3	90.3
National 300-7	81.3	91.4
Mulsoid	91.0	96.9
Control	99.0 <sup>6</sup>	99.3

\* The heavy line between fungicides indicates a significant difference with odds of 100 to 1, or greater.

Table IV shows that the colloidal sulphur sprays are more toxic than the other preparations when tested on an equal sulphur weight basis.

*Toxicity independent of acidity.* Since certain theories relative to the fungicidal action of sulphur (15) have stressed the importance of acids as-

TABLE V

THE LACK OF CORRELATION BETWEEN THE TOXICITY OF SULPHUR DUSTS AND THE ACIDITY OF THEIR WATER EXTRACTS

Before Rain			After Rain		
Order of toxicity*	Dust	pH	Order of toxicity*	Dust	pH
1	Dritomic Kolodust	6.0 7.9	1	National 300-7	8.1
2	Sulpho-Tone National 300-7	7.2 8.1	2	Kolodust Aero Smoke Ansbacher-Siegle	7.9 5.0 8.8
3	Aero Smoke Ansbacher-Siegle	5.0 8.8	3	Dritomic	6.0
4	Niagara 200	6.9	4	Sulpho-Tone Niagara 200	7.2 6.9

\* From Tables II and III.

<sup>2</sup> U. S. Patent No. 1,550,650.

sociated with the sulphur, an attempt has been made to determine if there is any correlation between the toxicity of the dusts and the acidity of their water extracts. The water extracts were obtained by triturating 100 gram portions of the dust with 100 cc. of distilled water and filtering the mixture. The H<sup>+</sup> ion concentration of the filtrates were determined by means of the glass electrode (16).

The pH values of the water extracts of the dusts as compared to the order of toxicity of the latter both before and after rain is shown in Table V. It is apparent that there is no correlation between the acidity of the water extracts and the toxicity of the dusts. Evidently the cause of the variation in toxicity of the various dusts must be sought in some other property.

#### PARTICLE SIZE

The fact that samples of several "straight" sulphur dusts exhibit the same order of toxicity before and after rain, suggests that there is some fundamental physical property whose value determines the toxicity in these cases. The property can scarcely be a chemical one since the sulphurs in question contain only a small amount of impurities. The most obvious property to be considered is the average particle size, or the related value, the "specific particle number", that is, the number of particles furnished by unit weight of the material.

Blodgett (1) has discussed the desirability of using sulphur of small particle size, and has quoted the opinions of others to the effect that the greater the degree of fineness of the sulphur, the more perfect would be its distribution and the greater its adherence. The present authors have previously compared (15) the relative toxicity of fractions of a sulphur dust obtained by sieving and have found that the toxicity increases as the mean particle size of the fraction decreases. Young and Tisdale (18) have noted the superior adherence of fine sulphur particles, and May and Young (8) have studied the relation between rainfall and the removal of sulphur from dusted apple leaves in the field. Streeter and Rankin (11) have examined a number of commercial sulphurs and determined their particle size—distribution curves by a microscopic method and compared the results with those obtained by sieving.

#### METHODS OF DETERMINATION

The term "mean particle diameter" as applied to a non-uniform powder is indefinite without further qualification. As ordinarily used the arithmetic mean diameter is intended, that is, the value obtained by summing up all the diameters and dividing by the number of particles. Other average diameters have been used however, and the relationships between them have been discussed by Green (4), Perrott and Kinney (10), and others.



A number of methods have been developed for estimating the average size of powders, as well as for determining the size frequency distribution. Perhaps the most commonly used method is that involving the use of sieves. Sieving methods are, however, subject to disadvantages, many of which have been pointed out by Chamot and Mason (2) and by Streeter and Rankin (11). Sieves are not available for classifying the very fine particles, which are usually the most important in the powder under consideration. The results obtained vary with the method of sieving adopted, and are affected by moisture, and by electrification of the powdered material, particularly in the case of sulphur. Sedimentation methods have been used, which depend on the relative rates of fall of particles of various sizes in a liquid or gaseous medium. They do not appear to be very well adapted, however, to the examination of sulphur. Direct microscopic measurement is also frequently used, and has been applied to dusting sulphur by Streeter and Rankin. In dealing with a fungicidal dust, the factor of chief importance is the distribution of the particles on the dusted surface, that is, the average number per unit area furnished by a given weight of the material, and the uniformity of distribution. A fairly rapid and simple method which permits the calculation of an average particle size, namely the diameter of the particle of average weight, can be based on microscopic counts of the number of particles lying within a known area of dusted surface, if the weight applied to this area is known. Such a method indicates the condition of the sulphur as actually used in dusting, and whatever aggregation or dispersion may occur during the dusting process will be that encountered in practice. In adapting this method to the present work, slides were carefully weighed on an analytical balance to the nearest 0.1 mg. An air damped balance<sup>3</sup> was found very useful, because of the rapidity with which weighings could be made. These weighed slides were then dusted, using the test tube duster previously described, and weighed again. In this way a series of dusted slides was obtained carrying from one-half a milligram to 20 milligrams of sulphur per slide. The slides were placed on a microscope stage and the image projected onto a ruled square subdivided into one hundred smaller squares. The side of each small square was 14 mm. which was equivalent to  $100\mu$  with the magnification used. For illumination a 6 volt, 108 watt research microscope lamp was used. Five fields were counted from each slide, thus the mean number of particles per  $100\mu$  square for a given weight of sulphur is based on a total of 500 counts per slide. The distribution of the sulphur particles on the  $100\mu$  squares was found to follow the well known Poisson series quite closely. If  $m$  is the average number of particles per square, then the fraction of squares having 0 particles is  $e^{-m}$ , 1 particle is  $me^{-m}$ , 2 particles is  $m^2e^{-m}/2!$ , and  $x$  particles is  $m^xe^{-m}/x!$ , etc.

<sup>3</sup> Type D 3, Sartorius-Werke, Göttingen, Germany.

Table VI gives the results of a typical series of observations, and shows the close agreement between the calculated and observed values. The fact that the distribution of the sulphur follows this series may be

TABLE VI  
THE ACTUAL DISTRIBUTION OF 2,463 SULPHUR PARTICLES ON A DUSTED SURFACE DIVIDED INTO ONE THOUSAND 100 $\mu$  SQUARES, AS COMPARED TO THAT CALCULATED FROM THE POISSON SERIES

Number of particles per 100 $\mu$ square	Per cent distribution of particles	
	Actual	Calculated
0 or more	100.0	100.0
1 " "	91.0	91.7
2 " "	69.9	69.8
3 " "	43.8	44.0
4 " "	24.0	23.3
5 " "	11.3	11.0
6 " "	4.5	4.3
7 " "	1.2	1.3
8 " "	0.5	0.3
9 " "	0.1	0.1

made use of to shorten the labor of counting. If the number of blank squares, containing no particles, are counted, then the average number per square may be obtained by calculation, or more readily by means of a chart given by Thorndike (12) provided the material is distributed according to the Poisson series.

In using the counting method for the purpose of determining the average particle size, counts were made of a number of slides carrying different weights of sulphur, for each brand under examination. The values obtained for particle number per 100 $\mu$  square were plotted against the weights in mg. of dust on the slide, and a straight line drawn through the points and passing through the origin. A typical graph is shown in Figure 2. The slope of this line  $n/W$  is related to the average particle size, and the relationship is given by the expression

$$\text{Mean particle diameter in } \mu = \sqrt[3]{\frac{10^9 \times 6Wa}{An\rho\pi}},$$

where  $W$  is the weight of dust on the slide in milligrams,  $A$  is the area of the slide in square microns,  $n$  is the average number of particles in the area  $a$  (in this case the area of a 100 $\mu$  square), and  $\rho$  is the density of sulphur. This is a special case of the more general formula given by Chamot and Mason (2, p. 233). The formula is derived from a consideration of the number of particles in a given weight of the dust from which the diameter of the particle of mean weight may be calculated under the assumption that this particle is spherical.

The results of particle size determinations for the four straight sulphur dusts are shown in Table VII. The mean diameters recorded in col-

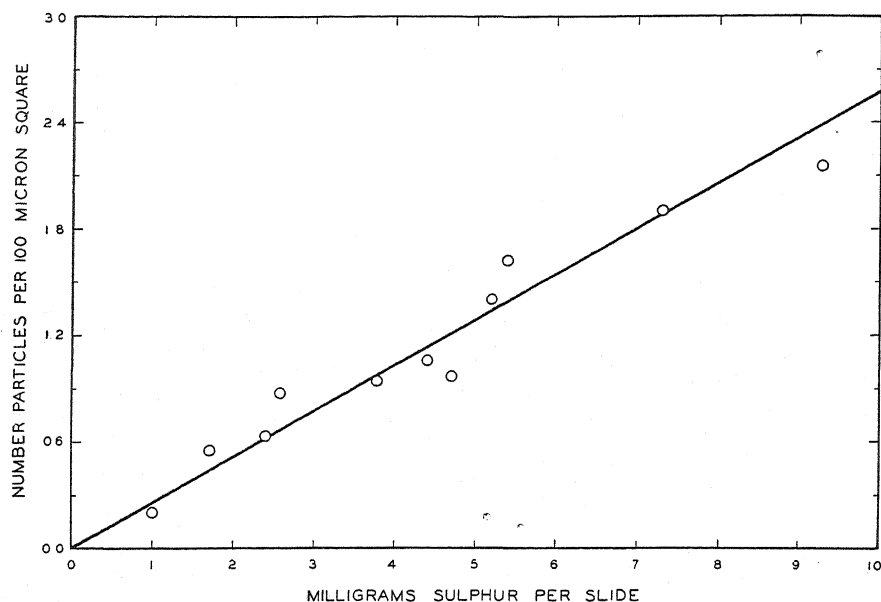


FIGURE 2. The relation between weight of sulphur and number of particles per given area for a typical sulphur dust. The mean particle diameter may be calculated from the slope of the line.

umn 3 are based on counts made at different times and using from 28 to 46 slides for each sample.

TABLE VII  
THE MEAN PARTICLE DIAMETER\* AND "SPECIFIC PARTICLE NUMBER" OF FOUR STRAIGHT SULPHUR DUSTS

Dust	Slope	Mean particle diameter $\mu$	Standard deviation		Mean number particles per mg. dust
			Mean	Single determination	
National 300-7	0.824	18.0	0.19	1.32	160,000
Aero Smoke	0.516	21.0	0.33	1.74	100,000
Ansbacher-Siegle	0.422	22.4	0.27	1.66	82,000
Niagara 200	0.258	26.5	0.34	2.36	50,000

\* Significant difference between all dusts with odds of greater than 100 to 1.

#### RELATION BETWEEN PARTICLE SIZE AND TOXICITY

It has been shown that the three dusts, National 300-7, Ansbacher-Siegle, and Niagara 200 differ significantly in particle size (Table VII) and

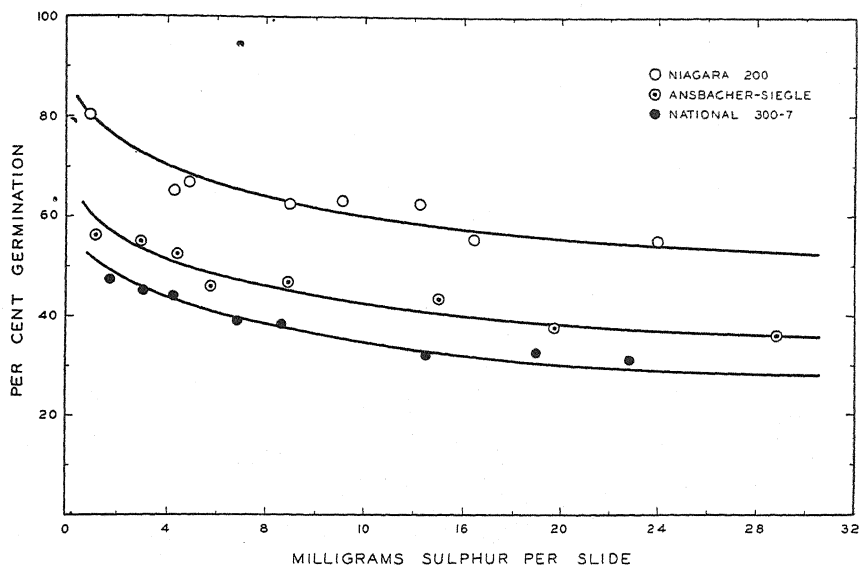


FIGURE 3. Toxicity curves, for three straight sulphur dusts of significantly differing mean particle size, plotted on a weight basis and showing a significant difference in toxicity. Germination of conidia of *Sclerotinia americana*.

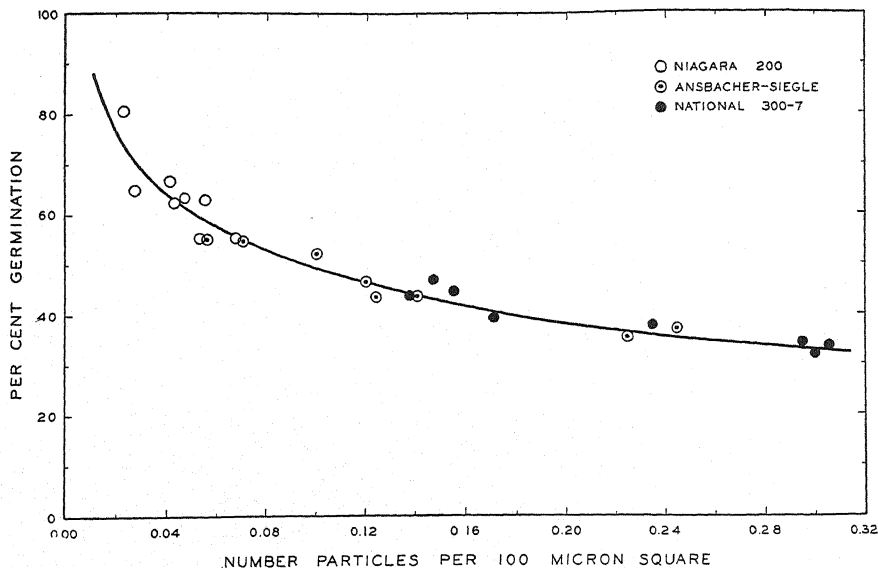


FIGURE 4. The three curves of Figure 3 plotted on the basis of particle numbers become one curve showing that the distribution or number of sulphur particles determines the toxicity.

in toxicity, both before and after rain (Tables II and III). A detailed study of these three straight dusts, relative to each other, has been made and the results are shown in Figures 3 and 4 and Table VIII.

Toxicity curves for the three dusts, following the rain test, were obtained using 14 day old spores in order to magnify the sensitivity of the spores to the dust. The tests were performed in duplicate, that is, two slides apiece were dusted with the various amounts of sulphur, separately weighed and the number of particles per unit area determined. Each point on the curves is representative of the mean of these duplicates and is based on counts of 600 spores. In Figure 3 the results are plotted on the basis of original weight of sulphur. It is apparent that the three dusts are significantly different and that the dust having the finest particles is the most toxic while that with the coarsest particles is the least toxic.

In Figure 4, however, the results of the same tests are plotted in terms of actual number of particles left per unit area after the rain test. It is seen that the three distinct curves of Figure 3 have become one and the same curve in Figure 4. Thus the toxicity of straight sulphur dusts is not dependent on some chemical characteristic, but is dependent on particle size because this property governs the number of particles in a given quantity of dust. That is, the relative toxicity of sulphur dusts is dependent on the number of sulphur particles and that dust furnishing the greatest number of particles per unit amount will be the most toxic.

TABLE VIII

SULPHUR DUSTS OF DIFFERENT PARTICLE MEAN DIAMETERS SHOWING NO DIFFERENCE IN TOXICITY WHEN APPLIED TO GIVE THE SAME NUMBER OF PARTICLES PER UNIT AREA\*

Dust	Mean particle diameter $\mu$	Weight of dust per slide in mg.	Number of particles per 100 $\mu$ square	Per cent germination <i>Sclerotinia americana</i>
Niagara 200	26.5	11.7	2.3	52.4
Ansbacher-Siegle	22.4	4.5	2.3	52.1
National 300-7	18.0	3.0	2.3	54.2
Control	—	—	—	97.6

\* No rain treatment.

Similar results and curves have been obtained without subjecting the dusts to the rain test. In Table VIII there is shown the results of toxicity tests in which the slides were dusted to give an approximately equal distribution of particles per unit area. It is seen that there is no significant difference in toxicity in the three dusts which, however, do differ significantly in particle size, in weight and in surface, the last being directly proportional to the square of the diameter.

To demonstrate further that relative toxicity is not an inherent quality

of a brand of sulphur dust, a fine fraction of Niagara 200, the coarsest dust, was obtained by air-flotation. The toxicity of this fine fraction was compared on an equal weight basis with that of the original dust both before and after the rain test, and with the finest dust, National 300-7, after the rain test. The results are given in Table IX and show that the sample with the smallest mean particle diameter, regardless of its origin, is the most

TABLE IX  
THE COMPARATIVE TOXICITY\* WITH AND WITHOUT THE RAIN TREATMENT OF A FINE FRACTION OF THE COARSEST SULPHUR DUST WITH THAT OF THE ORIGINAL DUST AND OF THE FINEST SULPHUR DUST

Dust	Without rain test		With rain test	
	Mean particle diameter $\mu$	Per cent germination <i>Sclerotinia americana</i>	Mean particle diameter $\mu$	Per cent germination <i>Sclerotinia americana</i>
Niagara 200	26.5	63.1	26.5	90.3
Fine Fraction of Niagara 200	16.9	46.0	15.1	74.5
National 300-7	—	—	18.0	80.5
Control	—	98.8	—	97.5

\* Between all pairs a significant difference with odds greater than 100 to 1.

toxic. That is, the fine fraction from the coarse dust is more toxic than the finest dust. The dust with the smallest particles furnishes the greatest number of particles per unit weight and is therefore the most toxic.

In the case of the two colloidal sulphurs, which were found to be more toxic than any other preparation tested, determinations of mean particle size were made by a method similar in principle to that used for the sulphur dusts, but with the use of dark field illumination. The values found were  $0.5\mu$  for Ialine, and  $2.4\mu$  for Premier Mill, which is in agreement with their relative order of toxicity.

#### RELATION OF PARTICLE SIZE TO ADHERENCE

The importance of mean particle size in relation to the toxicity of sulphur dusts having been shown, it was considered of interest to study this property further and investigate the relation of particle size to adherence following the rain treatment. Accordingly experiments were performed on adherence to glass slides and to the upper and lower surfaces of leaves. An effort was made to apply comparable numbers of particles per unit area of surface since the tests were based on number of particles rather than weight of dust.

Typical curves illustrating the adherence to glass slides are shown in Figure 5 where the number of particles of dust per unit area before the rain treatment is plotted against the per cent left after the treatment, for

three different dusts. It is seen that on an equal number basis there is a greater percentage of the particles of the finer dust left. Also it will be noted that as the original quantity of dust increases there is a marked decrease in adherence up to a certain point beyond which, however, the decrease lessens till the percentage adherence becomes practically constant.

The adherence tests with leaves included a study of three different dusts on the upper and lower surface of cherry leaves and in addition the adherence of a single dust to four different kinds of leaves since it might be expected that the nature of the leaf surface would be important in this respect. For this latter purpose leaves were chosen ranging from the rela-

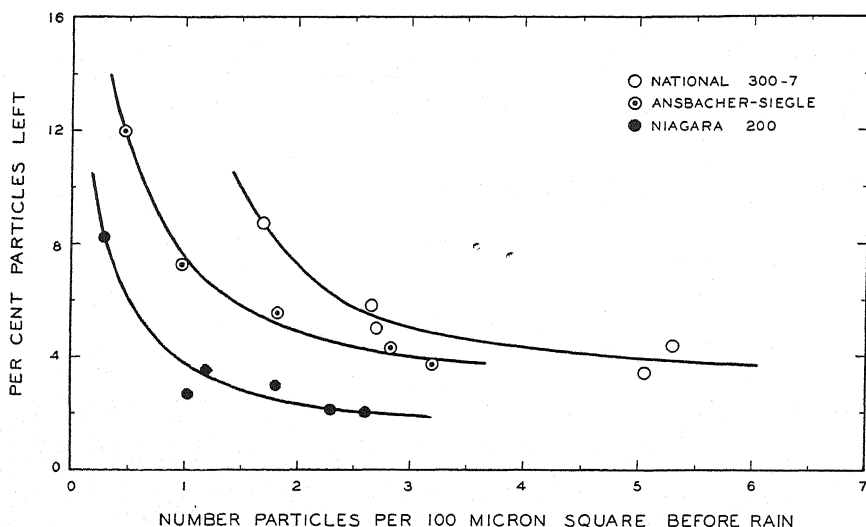


FIGURE 5. Typical adherence curves, for three straight sulphur dusts of significantly differing mean particle diameters, showing that the finest dust adheres best and that relative adherence decreases with an increase in the amount of dust applied.

tively smooth rhododendron (*Rhododendron* sp.) and cherry (*Prunus avium* L.) to the relatively hairy apple (*Pyrus malus* L.) and very hairy grape (*Vitis labruscana* Bailey). The leaves were dusted and subjected to the rain treatment in a similar manner to the slides. The distribution of sulphur particles on the leaf surfaces was determined by direct counting under the low power objective of the microscope using reflected light.

The results of the adherence test on cherry leaves with three different dusts are given in Table X. It was found that the adherence is greatest with the finer dusts and on the lower surface of the leaf. The cherry leaves are more hairy and irregular on their lower surface. By comparison with Figure 5 it will be seen that under the conditions of the experiment, the

adherence to glass slides is slightly less than to the upper surface of the cherry leaves. Table X shows further that it is the coarser particles which

TABLE X  
RAIN-ADHERENCE TESTS, ON UPPER AND LOWER SURFACE OF CHERRY LEAVES, OF  
SULPHUR DUSTS OF DIFFERENT MEAN PARTICLE DIAMETERS

Dust	Leaf surface	No. particles per sq. mm. before rain	No. particles per sq. mm. after rain	Per cent particles left	Mean particle diameter in $\mu$	
					Original dust	Dust left after rain
Niagara 200	Upper	73.5	4.8	6.5	26.5	18.6
	Lower	74.8	6.2	8.3		
Ansbacher-Siegle	Upper	64.6	6.7	10.4	22.4	15.4
	Lower	64.6	8.5	13.1		
National 300-7	Upper	71.5	10.0	14.0	18.0	13.3
	Lower	71.5	10.6	14.8		

are first washed away leaving on the leaf a dust of smaller mean particle diameter. The values representing mean particle diameter given in column 7 were necessarily obtained by direct measurement.

In Table XI it is shown that the adherence of sulphur dusts is greater on the hairy types of leaf surfaces. The adherence to the very hairy lower

TABLE XI  
PERCENTAGE OF NUMBER OF SULPHUR PARTICLES LEFT ON LEAVES AFTER RAIN TREATMENT

Leaf	70 particles per sq. mm. before rain		140 particles per sq. mm. before rain	
	Lower surface	Upper surface	Lower surface	Upper surface
Grape	25.4	16.8	15.5	9.9
Apple	44.5	7.7	35.2	6.2
Cherry	8.5	7.9	7.9	5.8
Rhododendron	9.8	7.5	6.7	6.5

surface of the grape and apple is to be noted. A large proportion of the adhering particles was found along the veins. This observation has also been made by Young (17), in the case of apple leaves. It is also evident, as was shown in Figure 5, that the adherence is relatively less when the original amount of dust is increased.

*Influence of electrical changes.* The exact nature of the forces which cause powdered substances to adhere to solid surfaces does not seem to be known. In the case of fungicides and insecticides some workers have considered that electrostatic changes on the particles may play an important part in promoting adherence to leaves (9). It may be easily demonstrated that sulphur dusted from a small test tube duster, or shaken



through cheesecloth, carries an electrical charge. It may also be shown that the removal of sulphur from a dusted leaf by blowing or shaking causes the leaves to become charged and also the sulphur. Whitman (14) has studied the electrification of dust clouds brought about by their forcible removal from surfaces, and has noted the fact that the sign of the charge on all the particles is not the same. He states further that the charge on a single particle quite often exceeds 20 electrons. It seemed to the present authors that it should be possible to determine whether or not electrostatic forces are concerned in the adherence of sulphur dust, by exposing a dusted surface to the influence of radium emanation (Radon). The ionizing radiation from this substance tends to eliminate electrostatic charges by making the air a conductor. It might be expected that under such conditions some of the sulphur dust would fall off the dusted surface. The experiment was performed using dusted tinfoil and a dusted leaf, suspended over sheets of glazed paper. When a small tube of radon was brought near the dusted surfaces, no effect whatever was observed on the dust, although the same tube brought near a charged gold leaf electroscope caused the leaves to fall together almost instantly. A tube containing radon was fastened to a glass slide which formed one of a series of slides which were dusted simultaneously. In this case also careful inspection failed to show that the radon had influenced the distribution or adherence of the dust in any way. It seems unlikely that such charges can account for the adherence of sulphur over any considerable period, particularly the resistance to washing away by rain.

#### DUSTING QUALITIES

An attempt to evaluate various sulphur dusts to be used as fungicides should include consideration of other properties besides the mean particle size. Among these is the property of flowing freely and dusting well which has not hitherto been expressed quantitatively.

Blodgett (1) has discussed this question and pointed out the desirability of using sulphur dusts which flow freely, and form a cloud of dust. There is a readily measurable quantity which seems to be related to the dusting properties of sulphur, namely the "angle of slope." This is the angle between the side and the base of a cone of dust which has been carefully built up by gently shaking the dust on a surface with a well defined raised circular border.

A study of the "angle of slope," furnished by many powdered substances, has been made by Langhans (5). This author gives a detailed description of the methods used by him for the determination and a discussion of the factors which influence its value.

In the present work, the sulphur dust was gently shaken through a funnel suspended over a circular steel disc 9 cm. in diameter. In this way a

cone of dust was built up, until further additions of dust led to no further increase in altitude of the cone. Examples of two such cones are shown in Figure 6. The altitude was then measured to the nearest millimeter, and

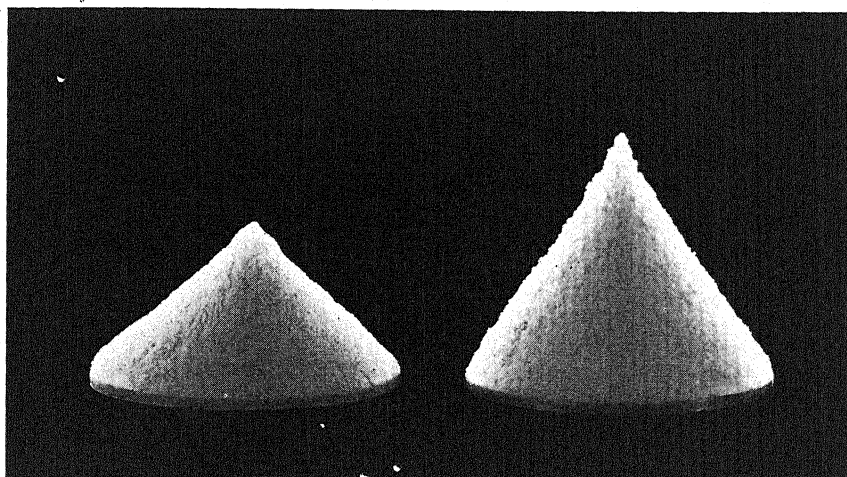


FIGURE 6. The "angle of slope" of sulphur dusts as a measure of dusting qualities. On the left a sulphur of good dusting qualities; on the right one of poor dusting qualities.

the tangent of the angle of slope calculated by dividing the altitude by the radius of the steel disc. The values obtained were somewhat variable in repetitions of the experiment on the same dust, but significant differences

TABLE XII  
THE DUSTING QUALITIES OF SULPHURS AS MEASURED BY THEIR "ANGLE OF SLOPE"

Dust	Mean tangent	Mean angle of slope	Standard deviation	
			Mean	Single determination
Sulpho-Tone	0.976	44° 18'	0° 16'	1° 05'
National 300-7 "Special"†	0.984	44° 32'	0° 30'	2° 08'
Kolodust	1.028	45° 48'	0° 25'	1° 37'
<hr/>				
Dritomic	1.261	51° 35'	0° 18'	1° 09'
<hr/>				
Niagara 200	1.375	53° 58'	0° 14'	1° 02'
Aero Smoke	1.386	54° 12'	0° 16'	1° 30'
<hr/>				
Ansbacher-Siegle	1.453	55° 28'	0° 12'	0° 56'
National 300-7	1.494	56° 12'	0° 14'	1° 15'

† National 300-7 plus 5% inert filler.

\* Heavy line between adjacent dusts indicates a significant difference with odds greater than 100 to 1.

were evident between dusts which were observed to vary widely in their dusting qualities. The results obtained in this study are shown in Table XII; and it may be seen that the dusts examined fall into four classes, which differ from each other significantly with odds greater than 100 to 1. The dusts which exhibit small angles of slope are those which flow freely and dust well, while those having large angles are inferior in these respects.

#### DISCUSSION

The experiments described in this paper have led to the conclusion that the mean particle size of sulphur dust is the most important single factor contributing to their toxicity. A small mean particle size is desirable for two reasons; in the first place, for a given weight, it involves a large number of particles, and hence great covering power. Secondly, small particles adhere better than large ones. A study of the curve shown in Figure 1 makes it appear that each sulphur particle has a definite radius of action, and unless every spore lies within this distance complete protection is not obtained. However, fine grinding of a dust is apt to furnish a product which cakes badly and does not exhibit satisfactory dusting qualities unless this tendency can be prevented by the addition of a suitable filler.

It appears to be possible to predict the relative toxicity of "straight" sulphur dusts by a determination of mean particle size and to estimate the dusting qualities from the angle of slope. However, in the case of "modified" sulphur dusts an actual determination of toxicity by means of spore germination tests, following the rain treatment, appears to be necessary.

#### SUMMARY

1. It has been found that many commercial sulphur fungicides differ significantly in toxicity to conidia of *Sclerotinia americana* when compared on an equal weight basis in laboratory tests. This difference cannot be ascribed to differences in the rate of formation of hydrogen sulphide in the presence of fungous spores. Neither can the difference in toxicity be correlated with the sulphur content nor with the acidity of the water extracts.

2. Sulphur fungicides may be classified primarily as dusts and sprays. Dusts may be subdivided into "straight" dusts and "modified" dusts, the latter containing materials intentionally added to modify their properties. Sprays may be designated as suspensions or as colloids depending on the degree of fineness of the particles.

3. The toxicity of representatives of these groups was studied both with and without being subjected to an artificial rain treatment. Before rain the modified dusts appear to be more toxic than the straight dusts, but after the rain test there is no difference between these two groups, although there is a significant difference between individual dusts. The col-

loidal sulphurs show a marked superiority to all other sulphur preparations following the rain test.

4. The order of toxicity of straight dusts is the same, before and after rain, and the toxicity increases with decrease in particle size.

5. A convenient method for the determination of the mean particle size of sulphur dusts is described. This method involves counting the number of particles furnished by a known weight of sulphur dusted on a surface of known area.

6. When straight sulphur dusts, whose particles differ significantly in their mean diameter, are compared on an equal weight basis, there are significant differences in toxicity. When, however, these dusts are compared on the basis of an equal number of particles per unit area, there is no difference in toxicity.

7. The adherence, following an artificial rain treatment, to glass slides and leaves, depends on the degree of fineness of the dust, the smaller particles being the most adherent. For a given dust an increase in the amount applied results in a decrease in the percentage adhering. The adherence to leaves is in general greater than that to glass slides, and increases with the roughness and hairiness of the leaf surface.

8. A useful measure of the dusting qualities of sulphur dust was found in the "angle of slope," that is, the angle between the side and the base of a cone of dust carefully built up to the maximum height attainable.

9. The most important single factor determining the toxicity of sulphur dust is the number of particles furnished by unit weight of the material. The greatest number of particles is furnished by the dust of smallest mean particle diameter as measured above, and the dust of smallest mean diameter is most adherent, and will give a denser distribution for a given weight. This relation between particle size and toxicity seems to hold within wide limits from relatively coarse particles down to those of colloidal dimensions. The dust having highest toxicity as determined by these tests is not necessarily the one showing the best dusting qualities.

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## SPECIFICITY OF THE PRECIPITIN REACTION IN TOBACCO MOSAIC DISEASE<sup>1</sup>

HELEN PURDY BEALE

### INTRODUCTION

Gruber and Durham (3) observed in 1896 that when a suspension of bacteria is mixed *in vitro* with serum from an animal that has been injected previously with cultures of the same microorganism, the individual bacteria are brought together in small clumps, i.e., agglutinated. These investigators also recognized the specificity of the reaction. The following year, Rudolf Kraus (6) demonstrated that cell-free culture filtrates of bacteria precipitate the serum drawn from animals that have received injections of the filtrate. While the early work of Kraus dealt chiefly with bacterial proteins, it was soon discovered that many different kinds of foreign proteins may cause a similar flocculation of the serum of animals into which they have previously been injected.

Practical application of these phenomena of specific agglutination and precipitation and of other antigen antibody reactions to the diagnosis of disease and the identification of proteins rapidly followed their discovery. Among the various uses to which these reactions have been put may be mentioned: the diagnosis of typhoid fever by the Widal agglutination test, the diagnosis and typing of Pneumococcus, the determination of the origin of blood stains in medico-legal practice, blood groupings necessary in blood transfusions, detection of food adulteration, and delicate tests for proteins present in so minute a quantity as to render their identification by the most skilled application of chemical methods exceedingly difficult.

While these serologic reactions have been employed successfully for over 30 years in the field of medicine, phytopathologists have made only a limited use of them in the study of plant diseases. The writer in 1928 decided to apply the technique of the serologic reactions to the investigation of tobacco mosaic disease in the hope of obtaining results that would throw some light upon the nature of the filterable virus responsible for this disease.

The results of former investigations by the writer (11, 12) have shown that when rabbits receive a series of injections of saline extracts of leaves from tobacco plants affected with the filterable virus of common field mosaic disease, the serum of these animals forms a heavy precipitate when

<sup>1</sup> Joint contribution of the Boyce Thompson Institute for Plant Research, Inc., Yonkers, New York, and the Department of Bacteriology, College of Physicians and Surgeons, Columbia University, New York, New York. These investigations were begun during the tenure of a National Research Council Fellowship in the Biological Sciences.

mixed with virus extract and incubated for a suitable period. No precipitate occurs, however, when the virus extract is added to serum drawn from a rabbit previous to injection of the plant juice. It has been shown further that precipitins to leaf extracts of healthy tobacco plants can also be produced in rabbits by the same method of hyperimmunization. Serum from animals injected with juice from healthy tobacco leaves forms a precipitate upon the addition of extracts of either healthy or diseased tobacco; antiserum for virus extract likewise contains precipitins to juice from healthy as well as from diseased tobacco leaves. However, the reaction differs noticeably in the amount of precipitate formed in the various combinations of the two antisera and plant extracts. The precipitate resulting from a mixture of juice from healthy tobacco and either antiserum is never heavy and rarely occurs in a saline dilution of antiserum exceeding 1 to 9. Likewise, in mixtures of virus extract and antiserum for juice from healthy tobacco leaves, only a slight precipitation occurs and only in low dilutions of antiserum. The largest amount of precipitate is produced in a mixture of virus extract and antiserum for virus extract, the homologous antiserum. Although the tobacco extracts are prepared so that the same quantity by green weight of healthy and diseased leaves is macerated in equal volumes of saline solution, the normal juice reacts with an antiserum of high precipitin titer for virus extract in a dilution of antiserum rarely exceeding 1 to 9, while virus extract may form a precipitate with the same antiserum in a dilution as high as 1 to 2000. The observation that virus extract reacts more readily with the homologous antiserum led to the assumption that there might be some substance present in virus extract not found in healthy tobacco leaf extract that is responsible for the reaction. Consequently, in the course of earlier investigations (11, 12) precipitin absorption experiments were undertaken in which the precipitins to normal tobacco extract were absorbed from the antiserum for virus extract by adding small amounts of juice from healthy tobacco leaves and incubating the mixtures for one and one-quarter hours at 37° C. After standing overnight in the ice chest, the mixtures were centrifugalized to throw down the precipitate. The supernatant fluid was then drawn off and more normal juice was added to the partially absorbed serum. The process was continued until no precipitate was formed upon the addition of normal juice, whereupon the absorption was regarded as complete. The amount of normal juice necessary for complete absorption of the precipitins was small, so that the dilution of the antiserum by the addition of normal juice is a negligible factor. The subsequent addition of virus extract to the antiserum from which all of the precipitins to normal juice had been absorbed resulted in the formation of a heavy flocculent precipitate. These results were interpreted as evidence of the presence of material in virus extract not found in normal juice and antigenic in nature, i.e., capable of stimulat-

ing the production of antibodies, in this instance precipitins, upon injection into an animal.

It was suggested that the antigenic material specific for virus extract and not present in extracts of healthy tobacco leaves might be either foreign protein or protein of the tobacco plant altered by mosaic disease, with a consequent change in its antigenic property. If foreign protein, two possibilities are open to consideration, the antigenic material might be virus itself or protein from microorganisms present in the mosaic diseased plant in the rôle of secondary invaders.

Matsumoto (8) using a similar method of hyperimmunization likewise produced precipitins in rabbits for virus extracts of tobacco affected with mosaic disease.

In earlier experiments (11, 12) it was also shown by the author that leaf extracts of tomato, pepper, and petunia, affected with the virus of common field mosaic disease, react with antiserum for virus extract of tobacco to form specific precipitates. Matsumoto and Somazawa (9) hyperimmunized rabbits separately for virus extracts of both tobacco and tomato. They then demonstrated by precipitin absorption experiments that all precipitins in either antiserum could be removed by complete absorption with the virus extract of either tobacco or tomato, thus proving the identity of the specific antigenic material in the case of both hosts of the virus.

It was thought that more evidence concerning the nature of the antigenic material specific for virus extract might be obtained by a further investigation of the specificity of the precipitin reaction. Three main lines of attack were planned. Only one kind of antiserum was used throughout the experiments, namely, the antiserum for virus extract of tobacco (*Nicotiana tabacum* L. var. Turkish) affected with common field mosaic disease. However, the source of virus extracts employed in the precipitin tests was varied. In one set of experiments leaf extracts were prepared from plants other than tobacco, affected with virus diseases distinct from tobacco mosaic. This source of virus extract was used on the assumption that if the specific antigenic material in tobacco virus extract were altered tobacco protein, similar substances might conceivably be found in different virus diseases as a result of a like alteration of normal host protein.

In another set of tests, leaf extracts of different hosts affected with the same virus, that of the common field mosaic of tobacco, were tested for the presence of material reacting with the specific precipitins in antiserum for tobacco virus extract. It was thought desirable to determine whether or not all hosts of the virus available for tests would give a positive precipitin reaction with the antiserum.

Finally, a set of experiments was undertaken in which two other viruses, distinct from that of tobacco mosaic, were multiplied separately in a com-



mon host, Turkish tobacco, which is also a host of tobacco mosaic virus. Leaf extracts of the common host affected with these different viruses were tested with the antiserum for tobacco mosaic virus extract for substances capable of reacting with the specific precipitins. By such a procedure it seemed likely that some evidence might be obtained relative to the nature of the antigenic material present in virus extract and not found in healthy tobacco leaves.

## EXPERIMENTAL PROCEDURE

### METHOD OF HYPERIMMUNIZATION

*Preparation of antigens.* All plant extracts were prepared by grinding green leaves to a pulp in a mortar with saline (0.85 per cent) solution. The macerated tissue was then removed by passage through coarse filter paper. A clear filtrate was then obtained by using a combination of Nos. 5 and 42 hard filter papers (Whatman).

*Injection of rabbits.* The method described in detail in an earlier article (12) was followed in general, except that in addition to the intravenous route for injection, the intraperitoneal was employed with equally good results. It was found that if the injections were discontinued for about 20 days following the first five injections given at two day intervals, the sixth injection after this rest period markedly increased the precipitin titer.<sup>2</sup> Similar results were obtained by giving each animal about three of these rest periods, the first one at the end of five injections and the second and third following two injections each. When the intravenous route was employed for hyperimmunization, only 0.5 cc. of antigen was administered at the first injection immediately following a rest period in order to avoid possible anaphylactic shock. The remainder of the antigen, usually about 1 cc., was injected an hour later.

*Collection of serum.* From 6 to 14 days after the last injection, the animals were etherized and bled to death aseptically from the carotid artery. The antiserum was allowed to separate from the clot overnight and the following morning inactivated for one-half hour at 56° C. (water bath) and stored in sterile tubes in an ice chest.

### PRECIPITIN EXPERIMENTS

Three-tenths cc. each of undiluted antigen and antiserum, undiluted or in a dilution of 1 to 4, were thoroughly mixed and the contents of the tubes were incubated for one and one-quarter hours in a water bath at 37° C. Upon removal from the water bath the tubes were examined for the presence of precipitate and placed in the ice chest overnight. The following

<sup>2</sup> This observation was made during a series of experiments carried out in collaboration with Mr. Joseph Webb.

morning a final record was made of the presence or absence of precipitate. The precipitin tests were repeated several times with each antigen employed. In every set of tests control tubes were included, some containing antigen with saline solution in place of antiserum, others having antiserum with saline solution substituted for antigen. In every case the antiserum for virus extract was used unabsorbed by normal tobacco juice inasmuch as any dilution of the antiserum, necessary in precipitin absorption tests, would be undesirable.

*Antiserum and extracts of different viruses in plants other than tobacco.* Sudan grass [*Holcus sudanensis* Bailey (*Andropogon sorghum* var. *sudanensis* Piper)], affected with the virus of sugar cane mosaic, and *Hippeastrum puniceum* Urban. (*H. equestre* Herb.), and *Lilium* species affected with their respective mosaic diseases, were selected as representative of virus diseases with which intracellular bodies resembling those found in tobacco mosaic disease are associated. In the case of these four maladies not only the macroscopic but the microscopic symptoms of the diseases bear a similarity to one another. Mosaic diseased plants of *Abutilon striatum* Dicks. var. *thompsoni* Veitch were chosen as typical of infectious chloroses transmissible by grafting, while yellows of peach (*Prunus persica* (L.) Stokes) was taken to represent another type of infectious chlorosis distinct from the mosaic type, but with symptoms sufficiently characteristic to include the disease in the class attributable by Kunkel (7, pp. 337, 338) and others to filterable viruses. In none of the maladies referred to, except tobacco mosaic, has actual filtration of the virus been demonstrated, but as Kunkel points out, they may be regarded as members of the group of diseases caused by viruses inasmuch as they produce a set of symptoms in their hosts characteristic of known filterable viruses and distinct from the symptoms resulting from infections caused by recognized plant parasites such as bacteria and fungi. In the case of many diseases included in the group due to filterable viruses, lack of ability to transmit the diseases mechanically has rendered proof of the filterability of the infectious agents impossible.

Extracts of these various plants, Sudan grass, *Hippeastrum*, lily, abutilon, and peach, affected with different viruses, were mixed with the antiserum for virus extract of tobacco affected with mosaic disease. After an incubation period of one and one-quarter hours the tubes were placed in the ice chest and on the following morning a record was made of the presence or absence of precipitate. The antiserum was used undiluted and in a dilution of 1 to 4. Tubes containing a mixture of antiserum and the homologous antigen were included in each set of experiments.

*Results.* Extracts of *Hippeastrum*, lily, and abutilon affected with the viruses of their respective mosaic diseases; Sudan grass, affected with the virus of sugar cane mosaic; and peach, affected with yellows, formed no

precipitate with antiserum for virus extract of tobacco mosaic disease (Table I).

TABLE I  
PRECIPITIN TESTS WITH ANTISERUM FOR TOBACCO MOSAIC VIRUS AND EXTRACTS OF  
VARIOUS PLANTS AFFECTED WITH DIFFERENT VIRUSES

Antiserum	Antigen		Precipitate*
	Host	Disease	
Undiluted (1:4) dil.	Sudan grass " "	Mosaic " "	o o
Undiluted (1:4) dil.	<i>Hippeastrum puniceum</i> " "	Mosaic " "	o o
Undiluted (1:4) dil.	Lily " "	Mosaic " "	o o
Undiluted (1:4) dil.	<i>Abutilon striatum</i> " "	Mosaic " "	o o
Undiluted (1:4) dil.	Peach " "	YeHows " "	o o
Undiluted (1:4) dil.	<i>Nicotiana tabacum</i> var. Turkish " " " "	" Tobacco mosaic " " "	++++ ++++

\* o = no precipitate; ± = very slight precipitate; + = slight precipitate; ++ = moderate precipitate; +++ = heavy precipitate; ++++ = very heavy precipitate.

*Antiserum and different plant extracts of tobacco mosaic virus.* It has been demonstrated previously by the writer (12) that extracts of tomato, pepper, and petunia affected with the virus of tobacco mosaic disease form heavy precipitates when added to the antiserum for virus extract from tobacco leaves. Matsumoto and Somazawa (9, pp. 229, 230) have further shown that the precipitins in antiserum for the virus extract of tobacco and tomato are identical. Other plants reported here have been added to the list of hosts of tobacco mosaic virus already tested for the ability to precipitate antiserum for virus extract of tobacco. Saline extracts of *Nicotiana glutinosa* L. and *N. rustica* L. affected with the virus were tested with the antiserum. All the hosts of tobacco mosaic virus so far reported as sources of virus in the precipitin tests are members of the Solanaceae; outside of this family only a few hosts, to which the virus has been successfully transmitted, are mentioned in the literature. Among them are bean (10), the crookneck squash (1), and *Martynia louisiana* (2). Bean and squash have not yet been tested but *Martynia louisiana* Mill., affected with the virus of tobacco mosaic, has been used as a source of virus extract to determine its ability to form a precipitate when mixed with equal parts of the antiserum. *Martynia* belongs to the family Martyniaceae, which is closely related to the Solanaceae.

*Results.* Leaf extracts of *Nicotiana rustica*, *N. glutinosa*, and *Martynia* affected with tobacco mosaic virus precipitated the antiserum for the same virus obtained from Turkish tobacco (Table II).

TABLE II  
PRECIPITIN TESTS WITH ANTISERUM FOR TOBACCO MOSAIC VIRUS AND EXTRACTS OF  
TOBACCO MOSAIC VIRUS ON VARIOUS HOSTS

Antiserum	Antigen		Precipitate*
	Host	Disease	
Undiluted (1:4) dil.	Tomato "	Tobacco mosaic " "	++++ ++++
Undiluted (1:4) dil.	Pepper "	" "	++++ ++++
Undiluted (1:4) dil.	Petunia "	" "	++++ ++++
Undiluted (1:4) dil.	<i>Nicotiana rustica</i> "	" "	++++ ++++
Undiluted (1:4) dil.	<i>Nicotiana glutinosa</i> "	" "	++ +
Undiluted (1:4) dil.	<i>Martynia louisiana</i> "	" "	++++ ++++
Undiluted (1:4) dil.	<i>Nicotiana tabacum</i> var. Turkish " " " "	" "	++++ ++++

\* See footnote in Table I.

*Antiserum and tobacco extracts of three distinct viruses.* Two virus diseases of Turkish tobacco distinct from tobacco mosaic were selected to test further the specificity of the precipitin reaction for a given strain of virus on the same host plant that was used for hyperimmunization. Ringspot disease and cucumber mosaic are both successfully reproduced in Turkish tobacco. On the basis commonly used at present for differentiation of the plant viruses, these can be identified as distinct from the virus causing tobacco mosaic disease. The distinguishing features, recently enumerated by Johnson and Hoggan (5, p. 29), are to be found in the symptom expression, the properties of virus extracts, the modes of transmission of the viruses, and the cytological picture.

Extracts of ringspot, cucumber and tobacco mosaic viruses multiplied separately in the common host, Turkish tobacco, were mixed with the antiserum to determine the presence or absence of substances reacting with the specific precipitins.

Another source of cucumber mosaic virus was utilized in these tests. *Nicotiana glutinosa* was employed for this purpose since it is a host common

to the viruses of both cucumber and tobacco mosaic diseases. While the tobacco mosaic virus remains localized in this plant, the cucumber mosaic virus produces a systemic infection in this host. Extracts of *N. glutinosa* affected with cucumber mosaic virus were tested with the antiserum for tobacco mosaic virus extract.

*Results.* Leaf extracts of Turkish tobacco affected with ringspot or cucumber mosaic disease gave only a slight precipitate with undiluted antiserum for tobacco mosaic virus on the same host and no precipitate with antiserum in a dilution of 1 to 4. Juice from *Nicotiana glutinosa* affected with cucumber mosaic virus produced only a slight precipitate with undiluted antiserum and no precipitate with antiserum in a dilution of 1 to 4 (Table III).

TABLE III

PRECIPITIN TESTS WITH ANTISERUM FOR TOBACCO MOSAIC VIRUS AND EXTRACTS OF TOBACCO AFFECTED WITH THREE DISTINCT VIRUSES

Antiserum	Antigen		Precipitate*
	Host	Disease	
Undiluted (1:4) dil.	<i>Nicotiana tabacum</i> var. Turkish " " " "	Ringspot " "	± o
Undiluted (1:4) dil.	<i>Nicotiana tabacum</i> var. Turkish " " " "	Cucumber mosaic " "	± o
Undiluted (1:4) dil.	<i>Nicotiana glutinosa</i> " "	Cucumber mosaic " "	± o
Undiluted (1:4) dil.	<i>Nicotiana tabacum</i> var. Turkish " " " "	Tobacco mosaic " "	++++ ++++

\* See footnote in Table I.

A heavy flocculent precipitate formed in every tube containing tobacco mosaic virus extract from Turkish tobacco and the homologous antiserum (Tables I, II, and III).

No precipitation occurred in any of the control tubes of antigen or antiserum.

#### DISCUSSION

By reference to Table III it will be noted that a slight precipitation occurs between mixtures of undiluted antiserum and every antigen prepared from tobacco. This is attributed to the presence of precipitins to normal tobacco juice. Leaf extracts of healthy Turkish tobacco, tomato, petunia, and pepper have been shown to precipitate antiserum for either healthy tobacco or tobacco virus extract. The reaction occurs only in undiluted antiserum or in dilutions usually not exceeding 1 to 4. When a

clear filtrate of a concentrated extract of healthy tobacco leaves is added to a 1 to 4 or a 1 to 9 dilution of an antiserum for tobacco virus having a high precipitin titer, a definite precipitate forms, while the same antigen added to normal rabbit serum gives an entirely negative reaction. Also virus extracts of Turkish tobacco react with low dilutions of antiserum for healthy tobacco juice to produce a slight precipitate, while normal rabbit serum fails to react. Therefore, there seems to be little doubt that when antigens are prepared from hosts identical to the one used for the purpose of hyperimmunization or from closely related hosts, a small part of the precipitate formed in mixtures of antiserum is due to precipitins to normal tobacco juice. The reaction can be readily eliminated by dilution of the antiserum and the use of a less concentrated antigen.

There has been no attempt from the evidence presented in this paper to prove that the viruses of cucumber mosaic and ringspot diseases may not be related serologically to the tobacco mosaic virus. Since a positive precipitin reaction is obtained only in mixtures of undiluted antiserum and antigen, any dilution of the antiserum necessary for obtaining proof by the use of precipitin absorption tests would be objectionable. Evidence of a possible relationship between these three viruses would seem to depend upon precipitin tests between the antiserum for virus extract of tobacco mosaic and extracts prepared from hosts of the ringspot and cucumber viruses unrelated to tobacco.

Owing to the possibility of the existence of a prozone phenomenon in the precipitin reactions, the antigens and antiserum were tested in various dilutions but only with negative results when the antiserum dilution exceeded 1 to 4, except in the case of the antigens from plants affected with the virus of tobacco mosaic disease. When undiluted antiserum of a high precipitin titer is used with the homologous antigen, the formation of a precipitate is frequently delayed although a flocculation eventually takes place.

As shown in Table II, the reaction between the antiserum and virus extract of *Nicotiana glutinosa* is recorded as moderate or in the case of diluted antiserum, only slight. The virus produces localized lesions in this host and according to Holmes (4, p. 52) the virus concentration is never great. Larger quantities of leaves of *N. glutinosa* extracted with a given volume of saline solution but having a low virus content, precipitate antiserum less than smaller amounts of the leaves of other hosts of the virus having a higher virus content.

Since microorganisms are frequently associated with mosaic diseased plants, the possibility that the specific precipitate may be due to such foreign protein should not be ignored. If the reaction were attributable to such concomitant antigens, the results would indicate that these microorganisms are present in every host of the virus thus far tested and are found

in tobacco, affected with the common field mosaic virus and not in tobacco affected with the viruses of either cucumber mosaic or ringspot disease.

#### SUMMARY AND CONCLUSIONS

1. Leaf extracts of Sudan grass, *Hippeastrum*, lily, and abutilon, each affected with its respective mosaic disease, and peach affected with yellows disease, were tested for their ability to precipitate antiserum for virus extract of tobacco mosaic disease. No precipitate occurred.

2. *Nicotiana glutinosa*, *N. rustica*, and *Martynia* were added to the list of hosts of tobacco mosaic virus previously tested with antiserum for the same virus in *N. tabacum* var. Turkish. The object was to determine the presence or absence of material reacting with the specific precipitins such as that already demonstrated in extracts of tomato, pepper, and petunia affected with the same virus. The presence of specific substances was demonstrated in every case.

3. The viruses of ringspot and cucumber mosaic diseases were multiplied in Turkish tobacco and leaf extracts of the affected plants were used in turn as antigens in precipitin tests with antiserum for tobacco mosaic virus extract of Turkish tobacco. A slight precipitation resulted in the tubes containing undiluted antiserum and virus extract such as occurs when juice from healthy tobacco is used with undiluted antiserum. No precipitate was demonstrable that was specific for virus extracts of tobacco affected with either ringspot or cucumber mosaic disease.

4. The conclusion is drawn that the results favor the interpretation that the specific antigenic substance in virus extract of tobacco mosaic disease is foreign antigenic material, possibly virus itself, rather than altered host protein.

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